THE EFFECTS OF PVP(FE(III)) CATALYST AND POLYMER MOLECULAR WEIGHT ON GENE DELIVERY VIA BIODEGRADABLE CROSSLINKED POLYETHYLENIMINE

BY

WING TAT VICTOR SHUM

THESIS

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Adviser:

Professor Daniel Pack

ABSTRACT

Human gene therapy has faced many setbacks due to the immunogenicity and oncogenity of viruses. Safe and efficient alternative gene delivery vehicles are needed to implement gene therapy in clinical practice. Polymeric vectors are an attractive option due to their availability, simple chemistry, and low toxicity and immunogenicity. Our group has previously reported biodegradable polyethylenimines (PEI) that show high transfection efficiency and low toxicity by cross-linking 800 Da PEI with diacrylate cross-linkers using Michael addition. However, the synthesis was difficult to control, inconsistent, and resulted in polymers with a narrow range of molecular weights. In the present work, we utilized a heterogenous PVP(Fe(III)) catalyst to provide a more controllable PEI cross-linking reaction and wider range of biodegradable PEIs. The biodegradable PEIs reported here have molecular weights ranging from 1.2 kDa to 48 kDa, are nontoxic in MDA-MB-231 cells, and show low toxicity in HeLa cells. At their respective optimal polymer:DNA ratios, these biodegradable PEIs demonstrated about 2-5-fold higher transfection efficiency and 2-7-fold higher cellular uptake, compared unmodified 25 kDa PEI. The biodegradable PEIs show similar DNA condensation properties as unmodified PEI but more readily unpackage DNA, based on ethidium bromide exclusion and heparan sulfate competitive displacement assays, which could contribute to their improved transfection efficiency. Overall, the synthesis reported here provides a more robust, controlled reaction to produce cross-linked biodegradable PEIs that show enhanced gene delivery, low toxicity, and high cellular uptake and can potentially be used for future in vivo studies.

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Chapter 1

Introduction

1.1 Gene Therapy in Medicine

Gene therapy is a treatment of human disease through the transfer of genetic material into specific cells within a patient [1]. For example, a therapeutic gene may be inserted into the genome of the targeted cells to replace an errant gene [2]. This is a promising technique for curing diseases that are caused by genetic mutations, which can lead to malfunction or deficiency of proteins. The causes of some common diseases like hemophilia, cystic fibrosis, and severe combined immunodeficiency (SCID) are related to specific aberrant genetic mutations [3-5]. Gene therapies are also being studied for conditions that are not directly attributed to a defective gene, such as Alzheimer's disease, cardiovascular disease, and cancer. Such treatments involve inserting genes to increase the production of specific proteins, to change gene expression, or to kill disease-causing cells by producing cytotoxic growth factors or proteins [6-9]. By genetically modifying patients' cells to produce therapeutic proteins, gene therapy has the potential to eliminate some problems associated with therapeutic drug use, namely poor bioavailability, high cost, and the necessity for frequent doses [10].

With the breakthrough of the Human Genome Project and the advancement of RNA and DNA technology, the particular human genes that correspond to different diseases or therapeutic protein production have been identified, and the promise of gene therapy has reached new heights. According to the National Institutes of Health, there are currently 1175 gene therapy clinical studies underway [11]. However, due to various limitations of delivering therapeutic RNA or DNA into target cells and/or tissues, gene therapy has yet to reach its full potential [12-13].

Currently, the most challenging aspect of gene therapy is to deliver a therapeutic gene into target cells and/or tissues efficiently and without significant side effects. Most current gene delivery methods suffer some critical drawbacks that plague new therapies from getting past the clinical trial development phase [14-15]. Much research is needed to better understand the cellular dynamics of gene delivery, and to design molecularly and clinically effective gene therapy procedures.

1.2 Ex Vivo Gene Therapy

One common method for introducing therapeutic genetic material into patients is ex vivo gene therapy. Ex vivo gene delivery involves extracting autologous cells from the patient's body, inserting a therapeutic gene into the cells, and transplanting the autologous cells back into the patient. This technique is highly patient-specific and requires surgeries to remove and implant the cells back into the patient. Ex vivo gene therapy has been tested in clinical trials as a potential treatment for patients suffering from adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID). Patients with ADA-SCID suffer from the lack of production of adenonsine deaminase, an enzyme that is required to breakdown and salvage purine, causing an accumulation of lymphocyte-toxic deoxyadenosine [16]. As a result, the buildup of ADA substrates affects the development of T cells and ultimately leads to a severely compromised immune system [17]. In September 1990, ex vivo gene therapy was used for the first time to treat a 4 year old girl with ADA-SCID in National Institutes of Health. In this clinical trial, the patient's T cells were extracted and infected with a retrovirus modified to contain the gene that encodes for properly functional ADA. The infected cells were transplanted back to the patient, and transgene expression was monitored for 2 years. After the treatment, the in vivo expression of the functional ADA gene was detected in the patient, which suggested ex vivo gene therapy was a viable potential treatment for the ADA-SCID [18]. In a separate clinical test, three

newborns who suffered from ADA-SCID were treated with ex vivo gene delivery by extracting their umbilical cord blood cells and infecting the cells with a retrovirus-containing gene that encoded for functional ADA [19]. Even though the transgene expression of ADA was detected in the patients' bone marrow, only low percentages of circulating blood cells were found to carry the inserted functional ADA gene, not enough to obtain the targeted gene expression levels or therapeutic effect.

Another somewhat successful clinical case using ex vivo gene therapy was the treatment of X-linked severe combined immunodeficiency (X-SCID). X-SCID is caused by a genetic mutation in the gene IL2RG that codes for gamma chain protein in various interleukin receptors. The mutation disables the protein and receptors, which in turn prohibits the lymphocytes from differentiation and maturation, resulting in a severely weakened immune system [20]. Patients with X-SCID often suffer from infections like pneumonitis and moniliasis due to their compromised immune system. In 2000, ten infant boys with X-SCID were treated with ex vivo gene therapy by harvesting bone marrow from each patient and transducing the cells with recombinant retrovirus containing a gene encoding for functional γ-cytokine receptor. Out of the ten patients, nine were cured of X-SCID, restoring their immune system. However, three of these patients were later diagnosed with leukemia [21, 22]. It was discovered that the cancer was caused by mutation of the retroviral insertion, in which the retrovirus inserted the gene for γ-cytokine receptor intro the patient's genome in the proximity of *LMO2* proto-oncogene promoter and led to the transcription and expression of *LMO2* [103] . These clinical cases show that gene therapy has great potential in curing genetic diseases, but at the same time, it also carries significant risks that must be eliminated for FDA-approval.

1.3 In Vivo Gene Therapy

In vivo gene delivery involves direct injection of therapeutic genetic material using either viral or non-viral vectors to target specific cells in the patient's body. Compared to ex vivo delivery, in vivo delivery does not require surgery to extract diseased tissues and, more importantly, allows therapeutic genes to reach more transient targets like moving cells. An example of a disease treated using in vivo gene delivery is cystic fibrosis (CF). CF is an inherited genetic disease that affects the lungs and digestive system, causing difficulty in breathing, lung infection, and often early death. The cause of CF is a mutation in the gene that is responsible for the production of cystic fibrosis transmembrane conductance regulator (CFTR), a protein found in the epithelial cells of the lungs and pancreas [23]. The genetic mutation of CFTR affects the movement of chloride and sodium ions in and out of the cells, resulting in an imbalance of ion concentration in the body and the formation of a layer of thick mucus that blocks the airways and causes lung infections [24]. Research has shown that to prevent the development of CF, only 5-10% of the normal gene expression of CFTR is needed [25]. Because of the low therapeutic efficiency needed to treat CF, in vivo gene therapy has become an ideal treatment option. Several clinical studies have been conducted using both viral and non-viral in vivo gene therapy to treat CF [26]. Approximately one-third of the patients recovered ~20% ion concentration [27]. Although much research is needed to increase in vivo gene therapy's efficacy, the initial success of these trials shows the potential of in vivo gene therapy as a safe alternative technique to treat genetic diseases.

Besides genetic diseases, another major potential treatment area for in vivo gene therapy is cancer. Currently, there are over 700 FDA-approved clinical trials using various in vivo gene therapies to treat tumors [28]. Cancer gene therapy can be summarized into 4 major approaches: 1) to replace the missing or altered gene that gives rise to cancer by substituting it with normal copies of the gene; 2) to activate the patient's immune system against cancer by inserting a gene that stimulates the patient's immune response; 3) to insert a gene into cancer

cells to make them more susceptible to radiation or chemotherapy; or 4) to induce the expression of a “suicide gene” in cancer cells that will trigger the patient’s immune response against them [29]. Regardless of approach, there are some barriers that need to be overcome in order for in vivo gene therapy to be used for cancer treatment. Researchers need to ensure that the gene is carried specifically to the cancer cells (not the surrounding healthy cells), is inserted into the correct location in the genome, and does not cause any harmful mutations.

1.4 Gene Delivery Methods

The key component of most gene therapy is to alter the target cells to produce the therapeutic protein to treat the particular disease. In order for gene therapy to work properly, the gene encoding for the corresponding protein must be delivered into the target cells’ nucleus for the gene to be transcribed and translated sufficiently. Two of the common physical methods used to directly inject DNA into a patient’s cells are electroporation and gene gun. For electroporation, the target cells are exposed to intense electric pulses, where the cell membranes are disrupted and permeabilized, allowing DNA to transport into the cells [30]. This method generally damages a large number of target cells, which makes it a non-ideal technique for gene therapy, though recent research has shown that high voltage electroporation in pigs can destroy target cells while surrounding cells remain unaffected [31]. Gene guns basically utilize helium propellant to shoot DNA-loaded gold nanoparticles into target cells [32]. Though efficient for in vitro experiments, gene guns require large physical force to shoot the gene into tissues, often causing tissue damage and rendering the technique non-ideal for in vivo experiments. Due to the physical damage that both of the above methods cause, the more commonly researched gene delivery methods involve encapsulating the therapeutic DNA in viral vectors (adenovirus, retrovirus, etc.) or non-viral vectors (cationic polymers, lipids, etc.) to transport the DNA into the target cells [33-34].

1.5 Viral Gene Delivery Vectors

A virus is a small infectious agent that is programmed to infect a living organism (the host) and hijack the host's cellular mechanisms to reproduce the virus molecule [35]. Generally, viruses consist of genetic material (DNA or RNA), a capsid protein coating that protects the genes, and/or a lipid envelope that protects the core when it is outside a cell. The two most common types of viruses used in gene therapy are retroviruses and adenoviruses. For retroviruses, the genetic material at the core is RNA, and the maximum size limit of the RNA is ~10 kbp. The capsid of a retrovirus is protected by a lipid envelope. The retrovirus, upon entering a host cell, reverse transcribes its RNA, integrates the corresponding DNA into the host's genome, and then replicates itself as part of the host's DNA. Unlike retroviruses, adenoviruses' genetic material is DNA, and the maximum size limit of the DNA is ~35 kbp. Adenoviruses are nonenveloped viruses, and its DNA is only delivered to the host's nucleus without incorporating into the host's genome. Adenoviruses are capable of infecting both dividing and non-dividing cells, while retroviruses can only infect dividing cells.

Due to viruses' natural ability to transfer genes into cells, they have become an ideal candidate for gene delivery vectors. However, all the potentially infectious genes and immunogenic capsid proteins must be removed from the viruses before they can be used for gene therapy. Once these genes and proteins are removed, therapeutic genes can be inserted into the viruses' capsid, creating an extremely efficient virus-based therapeutic gene delivery carrier. Unfortunately, it is difficult to remove completely all the potentially harmful components of a virus, and those that may remain behind pose a serious pathogenic threat to the patient upon administration. Even though viruses show promise as an efficient gene delivery vehicle, they still suffer many drawbacks, namely oncogenicity, immunogenicity, and lack of cell targeting capability that hinder them from being FDA-approved as a viable therapy.

1.5.1 Immunogenicity

As mentioned above, all viral genes and proteins must be completely removed in order for a virus to be used as a gene delivery vector. However, even if all viral components are extracted, a large dosage of viruses injected into a patient will still trigger the patient's immune system, resulting in either a serious immune response or greatly reduced gene delivery efficiency. The danger of fatal immune response caused by viral gene therapy was highlighted during a clinical trial in 1999 using an adenovirus as a delivery vector to treat ornithine transcarbamylase deficiency (OTCD) [36-37]. OTCD is a genetic disorder caused by the mutation of a gene that is normally responsible for the liver's production of ornithine transcarbamylase, an enzyme used to remove ammonia from the bloodstream. A large dose of adenovirus containing the therapeutic replacement gene was administered to the patient, causing an over-release of inflammatory cytokines and eventual systemic inflammatory response syndrome (SIRS). As a result of SIRS, the patient developed acute respiratory distress syndrome (ARDS), which proved fatal to the patient [38]. The death that occurred during this clinical trial led to a temporary suspension of all gene therapy trials by the FDA [39].

To prevent further immunogenicity-related deaths, researchers began to explore other types of viruses that possess less or no immunogenicity, such as adeno-associated viruses (AAVs). AAVs do not possess the pathogenicity associated with other types of viruses, and they currently have not been found to cause any human diseases [40]. AAVs are currently being tested in many clinical trials to treat various genetic diseases and prostate cancer [41]. There are a few limitations to using AAVs for gene therapy: 1) AAVs possess low cloning capability, making them difficult to mass produce, 2) AAVs have a small genome size (4.8kbp), which limits the type of therapeutic gene that could be inserted into the virus, and 3) AAV infection generally leads to an increase in antibody production, which could neutralize and reduce its efficacy [42-

43]. Regardless of the type of virus used for gene therapy, even if the viral vectors do not trigger a fatal reaction, any human immune response will greatly reduce the efficiency of the viral delivery vector by either eliminating the viruses before infection or killing the infected cells before the inserted gene is expressed [44].

1.5.2 Oncogenicity

One of the advantages of viral gene delivery is viruses' ability to carry genes efficiently into host cells. If the genetic cargo is inserted into the correct genomic location, viral gene therapy could provide the desired long-term therapeutic protein expression. However, if the gene is inserted randomly and disrupts normal gene expression or triggers an oncogene, cancer may develop as a result. Since adenoviruses do not insert their DNA into the host's genome, it is unlikely that adenoviruses will cause undesirable gene integration. AAVs do have the ability to integrate their DNA into the host's genome, but they primarily do so in a specific site on human chromosome 19 [45-46]. Unlike adenoviruses and AAVs, retroviruses present the threat of undesired gene deletion or activation and genetic mutation due to random DNA integration into the host's genome. Cancer-inducing retrovirus gene insertion was the cause of the leukemia development in the children treated for X-SCID mentioned previously. Besides gene integration issues, native components of the viruses themselves might also cause cancer under certain conditions [47-48].

1.5.3 Cell Targeting

In addition to delivering the therapeutic gene into the correct location in the cell's genome, another key component in gene therapy design is to have the vector delivered to the right type of cells. Even though all of the patient's cells contain the defective, disease-causing gene, only specific cells and tissues actually express the gene and need to be treated with gene therapy.

For example, OTCD is caused by a genetic mutation expressed in the liver; it would be desirable to design gene delivery vectors that would target specifically liver cells expressing the mutation in order to increase the efficiency of the treatment [49]. Viruses have evolved to be efficient at targeting specific cells, and one can enhance the cell targeting ability of viruses by using either transductional targeting or transcriptional targeting. In transductional targeting, ligands are attached to the surface of the viruses, or viruses are engineered to express the surface ligands, to target the receptors of specific cells [49-50]. In transcriptional targeting, the genetic material inside the viruses is altered to contain a promoter that will be triggered for transgene expression only if the virus is inside a specific cell type [51-52]. Even with detailed virus engineering, the native tropism of the viruses is intact, and the risk of the viruses infecting cells surrounding the target tissues remains.

1.5.4 Development and Production

The production of retroviral vectors for gene therapy involves two components: the retrovirus and the retrovirus packaging cell line. The retrovirus carries all the essential viral genes except the gene for viral proteins, which is provided by the retrovirus packaging cell line. By infecting the packaging cell line with retrovirus, one can produce retroviral vectors with desired properties or titer [104-105]. This production process has two major drawbacks: 1) the size of the gene that can be inserted into the retroviruses is limited to about 10 kbp and may not be appropriate for the therapeutic gene of interest; and 2) the expression of pathogenic viral proteins from any intact viral genes could trigger the host's fatal immune response. Because of these potential downfalls, a safer alternative virus production pathway involving gutted adenoviruses is being explored. In this case, the viruses only consist of the gene for packaging and replication. Larger therapeutic genes can be inserted into gutted viruses, reducing the risk of viral proteins due to

the removal of excess viral genes from the process. However, this process is more complex and labor-intensive compared to regular retrovirus production [53-55].

Most current virus production is targeted for lab bench-scale experiments. Both the packaging cell lines and purifications involved are expensive and difficult to scale up for mass production of viruses [56-57]. Without a more efficient and cost-effective method to mass produce viruses, viral gene therapy will remain a highly individualized treatment if approved.

1.6 Non-Viral Gene Delivery Vectors

Given the inherent properties of viral vectors in efficiently infecting various cell types and transferring genetic materials into the cell's nucleus, viruses have been extensively studied in clinical trials for gene therapy. However, as discussed above, viruses present many problems for in vivo gene therapy, including oncogenicity, pathogenicity, and cost. As a result, researchers have been exploring alternative materials that would provide similar gene delivery properties as viruses yet do not trigger immune responses from the patient and are relatively inexpensive to synthesize. Two such materials that are being studied to replace viral vectors are cationic lipids and polymers. The cationic surface charge of these non-viral vectors allows complexation with negatively charged DNA to form a shield to protect DNA from degradation. Some common lipids and polymers used in gene delivery research are shown in **Figure 1.1**. Unlike viruses, synthetic vectors need to overcome many barriers, both intracellular and extracellular, for successful transfection. A basic schematic of the process of non-viral gene delivery is shown in **Figure 1.2**. Much research has been invested into increasing the efficiency of non-viral vectors by improving their ability to bind to the cell surface and to escape cellular degradation mechanisms.

1.6.1 Advantages and Disadvantage of Non-viral Gene Delivery Vectors

One of the main advantages of using non-viral vectors over viral vectors in gene delivery is that non-viral vectors are generally non-immunogenic and non-pathogenic. More importantly, unlike viruses, synthetic vectors do not insert genes into the cell's genome, thus eliminating the risk of random insertion mutation. A summary of advantages and disadvantages of viral and non-viral vectors in gene delivery is listed in **Table 1.1**. Even though non-viral vectors show many good qualities as a gene delivery carrier, the main problem that plagues non-viral vectors from being a viable gene therapy option is its poor transfection efficiency compared to viruses. To understand this inefficiency in non-viral gene therapy, one needs to understand the cellular barriers and mechanisms involved in order for synthetic vehicles to deliver therapeutic gene into the cell's nucleus.

1.6.2 Extracellular Barriers

Unlike viruses, non-viral gene delivery vectors do not possess the natural ability of infecting cells and inserting genes into the host's nucleus. Polymer/DNA complexes (polyplexes) need to overcome many obstacles in order to reach and then enter the cells. For in vivo experiments, once the polyplexes are injected into the animal, they need to first withstand the degradative enzymes and serum proteins present in the blood stream. If cationic polymers do not condense the gene tightly or completely shield the gene from the enzyme, the gene will be digested, resulting in reduced delivery efficiency. Also, the serum protein in the bloodstream is negatively charged, which causes it to bind with the polyplexes that exhibit positive surface charge. If too much serum protein attaches to the polyplexes, the aggregates that form may be accumulated or filtered out by the liver or spleen [58]. One way to reduce serum protein aggregation is to shield the positive surface charge of the polyplexes from their surroundings by attaching biocompatible hydrophilic polymers like polyethylene glycol (PEG) [59-60].

Assuming the polyplexes have avoided the enzymatic degradation in the blood stream and reached the vicinity of the cells, they then need to attach themselves to the cells' surface in order to be internalized. The cationic surface charge of the polyplexes can naturally bind to the negatively charged cell surface through electrostatic interactions. However, this process is highly non-specific and leads to binding with surfaces of random cells instead of just the target cell type. Research has been done to attach various targeting ligands to the surface of the polyplexes to increase their targeting abilities. For example, transferrin and folate have been used as ligands to target the over-expressed receptors on cancer cells' surfaces to improve the efficiency of the non-viral gene delivery [61-62].

Due to their large size, the polyplexes cannot diffuse through cell membranes and must be internalized by a process calls endocytosis, where the cell membrane forms a vesicle around the polyplexes and pinches off into the cytosol. Endocytosis is generally classified into two groups: phagocytosis, for uptaking large particles, and pinocytosis, for uptaking smaller particles, fluid, and solutes. Phagocytosis happens in a limited number of cell types, where pinocytosis occurs in virtually all cells and is one of the most important pathways in gene delivery. There are four main types of pinocytosis: marcopinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin/caveolin-independent endocytosis (**Fig 1.3**). Each pathway will result in different sizes of vesicle and leads to different subsequent intracellular processes for the endocytosed polyplexes [63].

1.6.3 Intracellular Barriers

Once a non-viral gene delivery vector is internalized from the cell's surface via pinocytosis, it will initially be located within either a caveosome or an early endosome, for caveolin-mediated and clathrin-mediated pathways, respectively [64-65]. The clathrin-mediated pathway always leads to early endosomes, in which some will recognize the polyplex as a foreign object and

exocytose it. Otherwise, the early endosome will acidify and eventually become a lysosome and degrade the polyplex. For caveolin-mediated pathways, the caveosome remains at neutral pH and does not end up in lysosome. However, the fate of the caveosome is not well established. Evidence has suggested that caveosomes may merge into clathrin-mediated pathway in the early endosome stage or be localized into recycling endosomes [112-115]. Regardless of uptake pathway, the ability of escaping from the endsosome prior to lysosomal digestion is one of the most crucial factors that dictate the efficiency of a non-viral gene delivery system. For lipid gene delivery, it has been speculated that the cationic lipid mixes with the negatively charged lipids in the endosome, which causes a reduction in the lipid's DNA condensation strength and leads to the release of the DNA into the cytosol [66]. Unlike lipids, certain cationic polymers, like PEI, have a completely different proposed mechanism, called the proton sponge effect, for escaping endosomes. In general, ATPase on the endosomal membrane actively transports protons into the early endosome to acidify the endosomal environment. Due to the high density of secondary and tertiary amines on the polymer with pKa values between physiological and lysosomal pH, proton sponge materials like PEI prevent the acidification of the endosome by absorbing the protons through the protonation of their own amine groups. As a result, the ATPase transports more protons into the endosome to try to reach the target acidic pH. To retain electroneutrality, an increased amount of counter ions, mainly chloride, are also transported into the endosome, leading to an increase in internal osmotic pressure, endosomal swelling, and eventual burst (**Fig 1.4**).

After the polyplex escapes from the endosome, it must travel through the cytosol to reach the nuclear membrane. Studies have shown that random diffusion is unlikely to be responsible for moving the polyplex to the nuclear membrane [67]. Based on fluorescent particle tracking experiments, microtubules and filaments appear to actively transport the polyplex through cytosol using molecular motors [68-69].

One of the last intracellular trafficking barriers the polyplexes need to overcome after reaching the nuclear membrane is to gain entrance to the nucleus and unpack the gene inside the nucleus [70-72]. There are three possible methods the polyplexes use for nuclear entry. The first method involves the polyplexes utilizing the nuclear pores on the membrane for entrance. There are thousands of the tiny pores, ~150 nanometers in diameter, on the surface of the nuclear membrane that open or close depending on the presence of a nuclear localization signal (NLS) on the object trying to enter [106]. In their open state, the pores allow active transport of molecules with a diameter of ~26 nanometers, while in their closed state, the pores only allow molecules with diameters less than 9 nm to diffuse through [73]. As a result, research has been done to covalently attach NLS to DNA to induce the opening of the nuclear pores for active transport of DNA, and potentially entire polyplexes, into the nucleus [74-75]. The second nuclear entry method involves polyplexes entering the nucleus through the breakdown of the nuclear envelope during mitosis. As the cells split into two daughter cells, the nuclear envelope breaks open and allows the negatively charged chromatin to interact with the cationic polyplexes and to competitively displace DNA from the cationic polymers. The displaced DNA is then trapped inside the nucleus as the nuclear envelope is reconstructed after mitosis, allowing the DNA to be transcribed inside the host's nucleus. Studies have shown that gene delivery efficiency increases when cells are dividing [76]. With the understanding of the extracellular and intracellular barriers involved with non-viral gene delivery in mind, it is important to choose materials that condense and protect DNA, are able to be internalized by cells, escape the endosome, and carry the DNA to the nucleus. As mentioned earlier, two of the most heavily researched materials that fulfill all of these requirements are cationic lipids and polymers.

1.6.4 Lipid-Mediated Gene Delivery

Cationic lipids, like 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), dioleoylphophatidylethanolamine (DOPE), and N-[1-dioleyloxy)propyl]-N,N,N-trimethylammonium (DOTMA), are commonly used to complex with DNA to form lipoplexes for lipid-based gene delivery [107]. Cationic lipids consist of three major components: a hydrophobic lipid anchor group, a linker group, and a positively charged headgroup. The hydrophobic lipid anchor helps the formation of liposomes, the headgroup condenses the DNA, and the linker group dictates the stability of the lipids. For example, the ether linker between headgroup and acyl chain of DOTMA is more stable than the labile ester linkage of DOTAP [108]. In additional to the property of the linker group, the valence of the lipid headgroup has also been shown to affect the lipid's gene transfer capability [109-110]. The overall transfection efficiency of lipoplexes is very low compared to viruses due to the intracellular barriers mentioned above. . Researchers have explored the addition of helper lipids and targeting ligands to lipoplexes to facilitate endosomal escape and to increase cell targeting and uptake. Both strategies have shown an improvement in transfection efficiency [77-79].

1.6.5 Polymeric Gene Delivery

Cationic polymers, like poly-l-lysine (PLL), polyamidoamine (PAMAM) ,polyethylenimine (PEI), poly(β-amino ester)s, and poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA), are heavily studied as potential gene delivery vehicles due to their capability to condense negatively charged DNA and protect the DNA from its cellular surroundings. Poly(β-amino ester)s have been shown to mediate gene transfer better than Lipofectamine 2000, a commercial lipid-based gene delivery reagent [111], However, unlike poly(β-amino ester)s, other cationic polymers are plagued by either low transfection efficiency (PLL, pDMAEMA) or high toxicity (PEI and PAMAM), making them non-ideal gene delivery vectors [80-82]. Similar to lipoplex gene delivery, many researchers have investigated different synthesis strategies to increase the

efficiency of these non-viral vectors via aiding the endosomal escape, lowering the toxicity, or improving cell targeting. For example, melittin, an endosomolytic peptide, is added onto PLL polyplexes to aid their endosomal escape capability [83]. Polyethylene glycol (PEG) has been added onto PAMAM to shield the positive surface charge from the cells, reducing toxicity and increasing efficiency [84].

Compared to other cationic polymers, PEI is one of the most commonly used in gene delivery. In addition to its ability to condense DNA, it has also shown relatively high transfection efficiency in vitro. PEI is cheap and commercially available in linear or branched form in different molecular weights from 0.8 to 1000 kDa. It has high primary, secondary, and tertiary amine densities, which allows easy functionalization of PEI with ligands to enhance its cell targeting and uptake [85-87]. The 25-kDa unmodified form of PEI has been shown to have the highest transfection efficiency in vitro but its high toxicity prevents it from being used clinically. As a result, it is commonly used as a transfection reference standard to compare with other newly synthesized gene delivery vectors. Much research has been done to reduce the toxicity of PEI while improving its efficiency and cellular uptake by modifying or functionalizing the polymer's structure. One of the main strategies being used to achieve this goal is to cross-link low molecular weight PEI, which is relatively non-toxic, with a degradable cross-linker.

1.7 Biodegradable Cross-linked Polyethylenimine

In general, gene delivery efficiency is directly related to the molecular weight of PEI, while toxicity is inversely related. Gosselin et al. concluded that 25 kDa PEI displays gene delivery efficiency 50-fold higher than 800 Da PEI but reduces cell viability by more than half [88]. Since material toxicity should be one of the major considerations in any gene delivery vector's design, researchers have explored various alternatives to temporarily increase the molecular weight of low molecular weight cationic polymers. The resulting polymers would condense DNA and

possess gene delivery efficiency similar to their high molecular weight counterparts and degrade into a less toxic low molecular weight version of the polymers inside the cells. One common approach is to insert a degradable cross-link between low molecular weight cationic polymer molecules. Many syntheses have been investigated to cross-link polymers using different cross-linker-polymer combinations. Petersen et al. have synthesized a biodegradable PEI derivative by linking PEI with oligo(L-lactic acid-*co*-succinic acid) (OLSA, 1000 Da) (**Figure 1.5**). The resulting polymer displays 10-fold increases in transfection efficiency and minimal toxicity compared to 25 kDa PEI [89]. Another version of degradable PEI is synthesized by linking 1.8 kDa PEI and glutadialdehyde with acid-labile imine linker that shows 30% improvement in toxicity but displays only about 80-90% of 25 kDa PEI's transgene expression (**Figure 1.5**) [90]. These results indicate that the cross-linking design is crucial for synthesizing degradable synthetic gene delivery vectors that show both low toxicity and high efficiency.

1.7.1 Disulfide Cross-linked Polymers

One of the specific cross-linking strategies that have been commonly used in peptide, PLL, PEI, and other polycations is the formation of disulfide cross-links. Because disulfide bonds will cleave under reducing conditions, the high molecular weight cross-linked materials will revert back to their original low molecular weight form inside the cytoplasm, aiding the release of DNA and reducing overall toxicity. McKenzie et al. showed that a disulfide cross-linkage between peptides increases the buffering capacity of the peptide/DNA complexes and improved gene delivery efficiency (**Figure 1.6**) [91]. By combining PEG and PLL to form block cationic polyplexes through disulfide bonding, Miyata et al. improved the stability of the polyplexes and increased transfection efficiency by at least 100-fold compared to PLL alone (**Figure 1.6**) [92]. Peng et al. reported a disulfide cross-linked PEI prepared by thiolation of 800 Da PEI that shows comparable transfection efficiency and improved toxicity compared to 25 kDa PEI (**Figure 1.6**)

[93]. Unfortunately, not all polymers with disulfide linkages show an increase in transgene efficiency. Gosselin et al. have disulfide cross-linked 800 Da PEI with dithiobis(succinimidylpropionate) (DSP) and dimethyl·3,3‘-dithiobispropionimidate·2HCl (DTBP). The resulting polyplexes only improve the toxicity but cannot outperform 25 kDa PEI in transgene expression (**Figure 1.6**) [88].

1.7.2 Diacrylate Cross-linked PEI

Another strategy used in synthesizing degradable polycations is the Michael addition of amines to acrylate groups. Lynn et al. screened a library of 140 diacrylate cross-linked amine polymers, showing some specific combinations of diacrylates and amine monomers with promising transgene expression [94]. Since then, many researchers have studied further the gene delivery properties of those polymers reported by Lynn et al. Forrest et al. detailed the synthesis of degradable PEI derivative by cross-linking 800 Da PEI with 1,3-butanediol diacrylate and 1,6-hexanediol diacrylate. The resulting polycations showed two- to 16-fold increase in transfection efficiency compared to 25 kDa PEI, and are essentially non-toxic [95]. Using a similar reaction scheme, Dong et al. synthesized cross-linked PEI with 800 Da PEI and 1,4-butanediol diacrylate and reported that the new polymer mediates comparable gene expression to 25 kDa PEI in vitro but not in vivo [96]. To further the study of Lynn et al., Thomas et al. used high-throughput synthesis and screening to test the in vitro and in vivo transfection efficiency of 24 bi- and oligo-functional acrylates separately cross-linked with linear 423 Da PEI and branched 1.8 kDa PEI [97]. They concluded that tricyclo [5.2.1.0] decame-dimethanol diacrylate cross-linked PEI derivative shows 3,600-fold improvement in transfection over the non-cross-linked 423 Da PEI in vitro, and mixed PEIs (423 Da PEI plus 1.8 kDa PEI) cross-linked with propylene glycol glycerolate diacrylate mediates 53-fold higher gene expression than the non-cross-linked version in vivo. In addition to transfection and toxicity studies, research has also been done to

characterize the degradation of diacrylate cross-linked PEI derivatives using real time ^{1}H NMR and Monte Carlos simulations [98].

1.7.3 PVP(Fe(III)) Catalyzed Biodegradable PEI Synthesis

As discussed above, Forrest et al. first reported the synthesis of degradable PEI by cross-linking low molecular weight PEI with diacrylates. The resulting PEI derivatives show minimal toxicity and increased gene delivery efficiency compared to 25 kDa PEI [95]. However, the synthesis was reported to be difficult to control and inconsistent. In this project, we investigated a different synthesis of the degradable PEI derivatives reported by Forrest et al. by utilizing poly(4-vinylpyridine) (PVP)-supported Fe(III) heterogenous catalyst. Fe(III) has the ability to catalyze the diacrylate cross-linking reaction, but separating it from PEI post-synthesis is difficult due to PEI's metal chelating potential [99]. By incorporating polymer support, the PVP(Fe(III)) heterogenous catalyst provides a low cost catalyst that catalyzes the synthesis of degradable PEI and allows easy catalyst recovery [100]. As a result, this alternative synthesis scheme provides a more controllable PEI cross-linking method and degradable PEIs with wider range of molecular weights. In the following chapters, we detail the synthesis and characterization of the degradable PEIs, involving catalysis by PVP(Fe(III)) for cross-linking of 800 Da PEI with 1,6-hexanediol diacrylate. These degradable PEIs' molecular weights range from 1.2 kDa to 48 kDa, and these materials also show higher transfection efficiency and cellular uptake, while showing minimal toxicity, compared to unmodified 25 kDa PEI in HeLa and MDA-MB-231 cell lines.

1.8 Tables and Figures

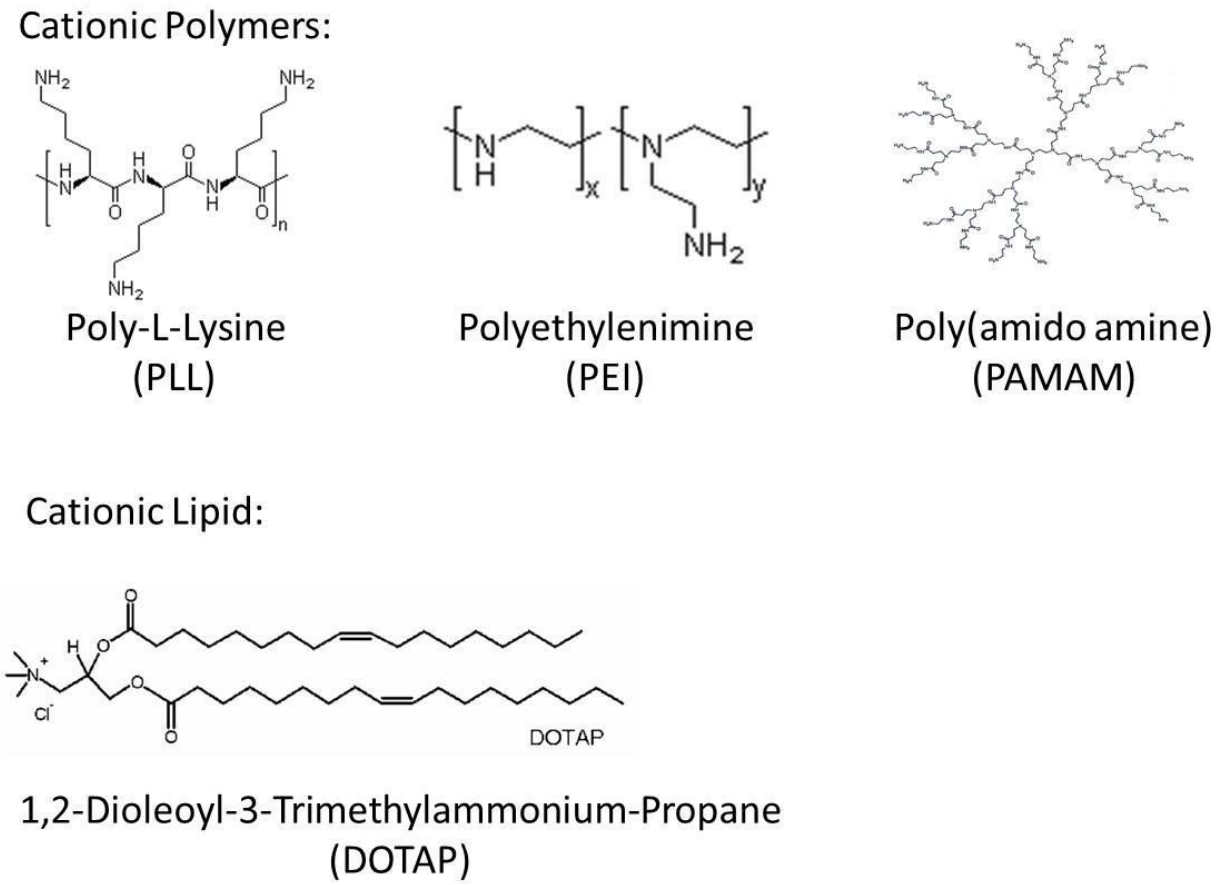

Figure 1.1: Commonly used cationic polymers and lipid for non-viral gene delivery [116]

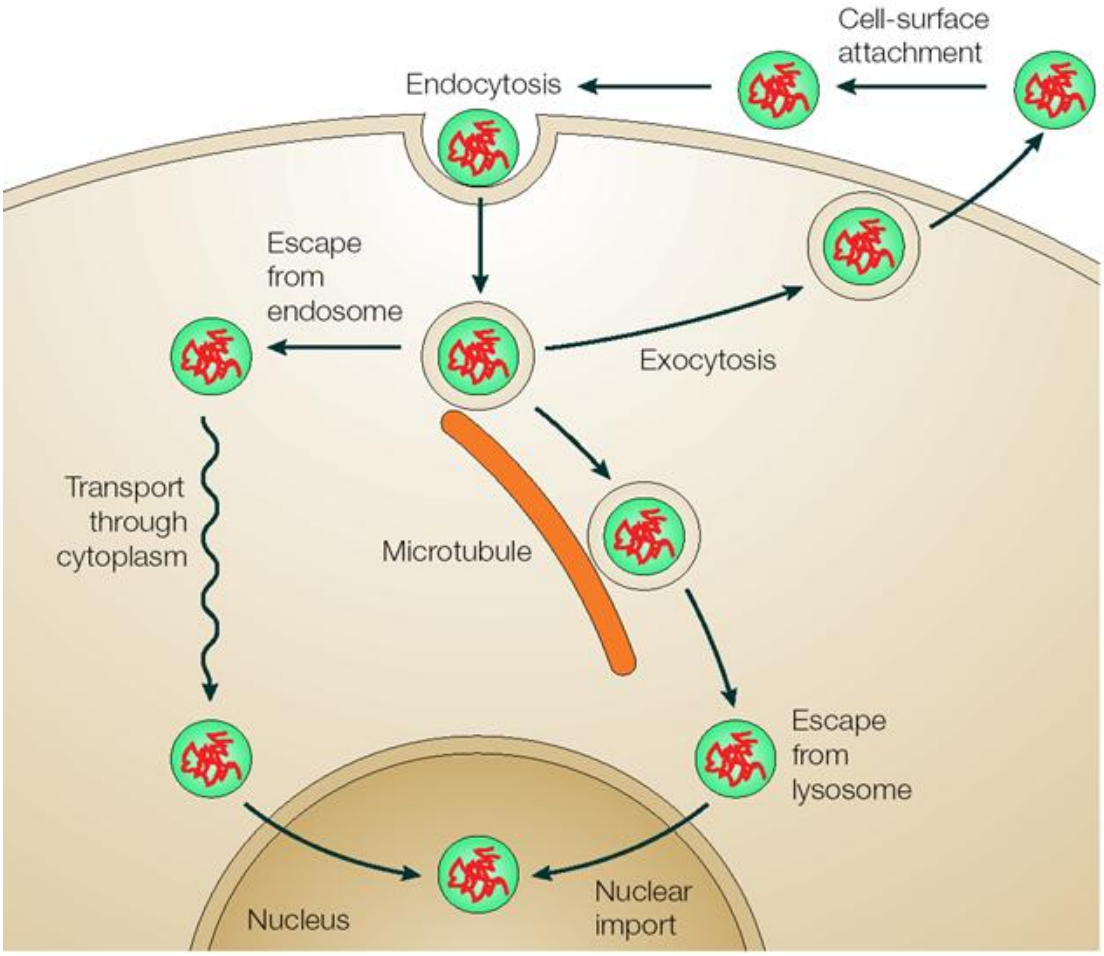

Figure 1.2: Cellular mechanisms involved in non-viral gene delivery. Complexes must overcome many barriers, including cell surface binding, endocytosis, exocytosis, lysosomes, endosomes, nuclear entry, and DNA unpackaging, in order to carry the therapeutic gene into the host's nucleus for transfection [101]

Table 1.1: Advantages and disadvantages of viral and non-viral vectors for gene therapy

	Viruses	**Non-Viral Vectors**
Immunogenicity	High	Low
Pathogenicity	High	None
Oncogenicity	High	None
Toxicity	Low	High
Efficiency	High	Poor
Cell Targeting Ability	Difficult to Adjust	Easy to Modify
Availability	Low/Custom Made	Readily Available
Cost	Expensive	Cheap

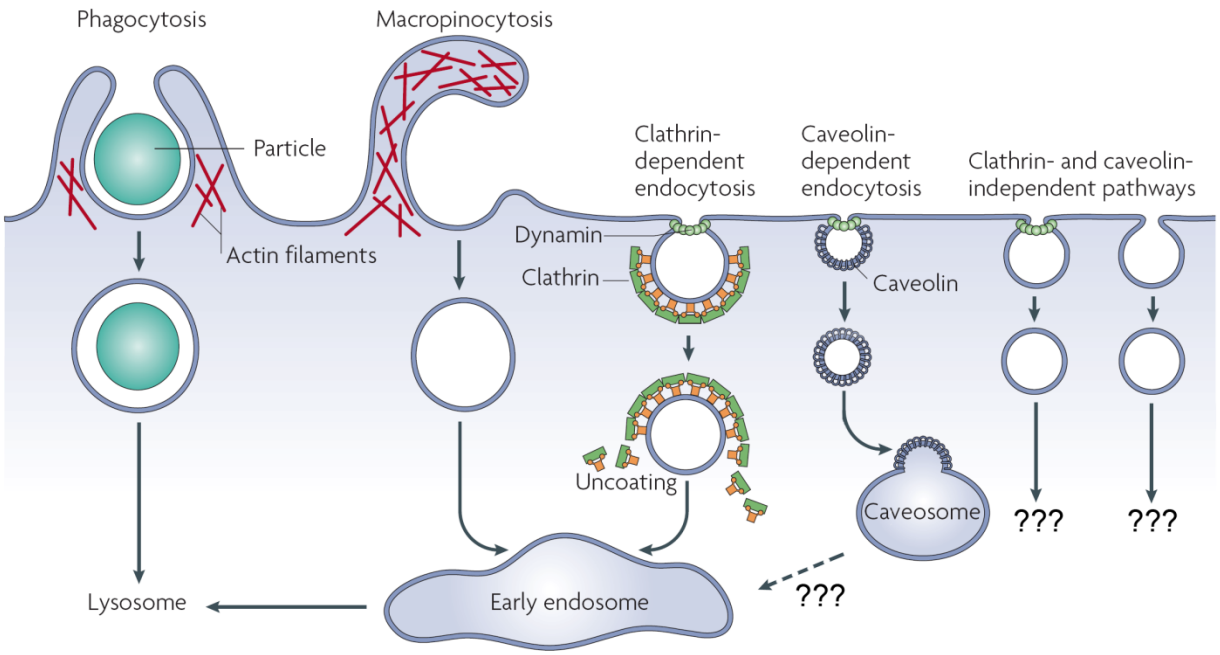

Figure 1.3: A simplified schematic summarizing various endocytic pathways into cells [102]

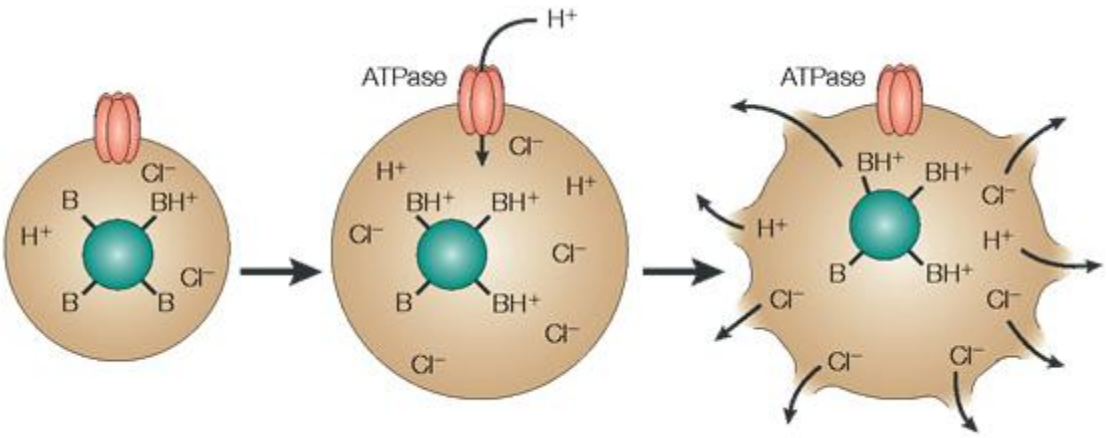

Figure 1.4: Schematic of endosomal escape through proton sponge mechanism [101]

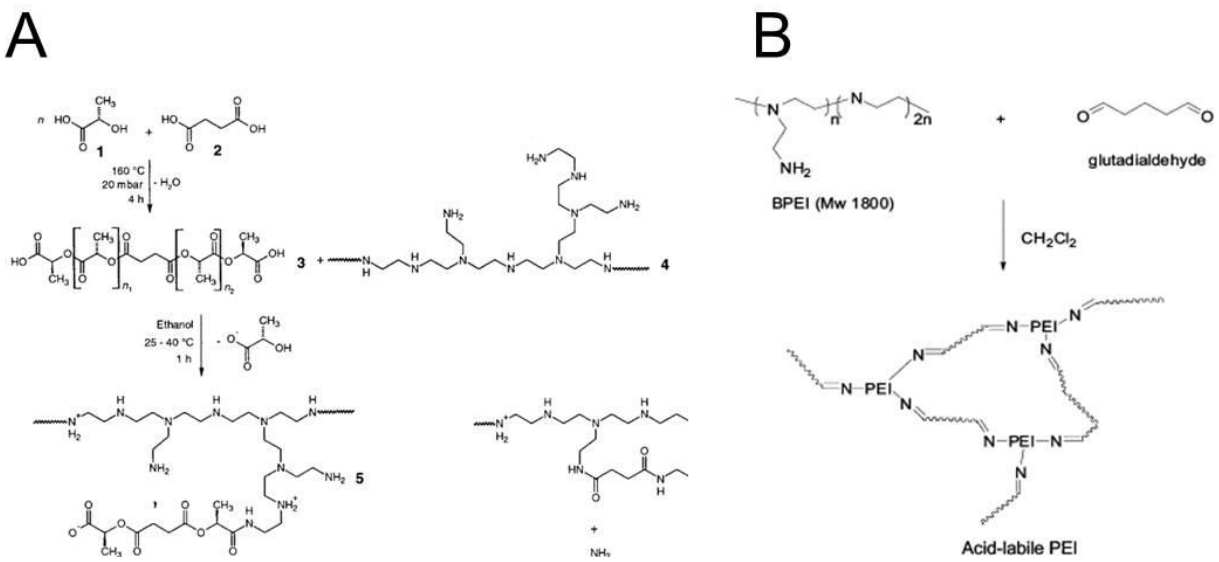

Figure 1.5: Reaction schematics of various cross-linked polymers. A) Petersen et al. synthesized biodegradable vector by cross-linking 1.2 kDa PEI with oligo(L-lactic acid-co-succinic acid) (OLSA) [89] and B) Kim et al. used acid-labile imine linker (glutadialdehyde) to produce a biodegradable PEI gene carrier [90]

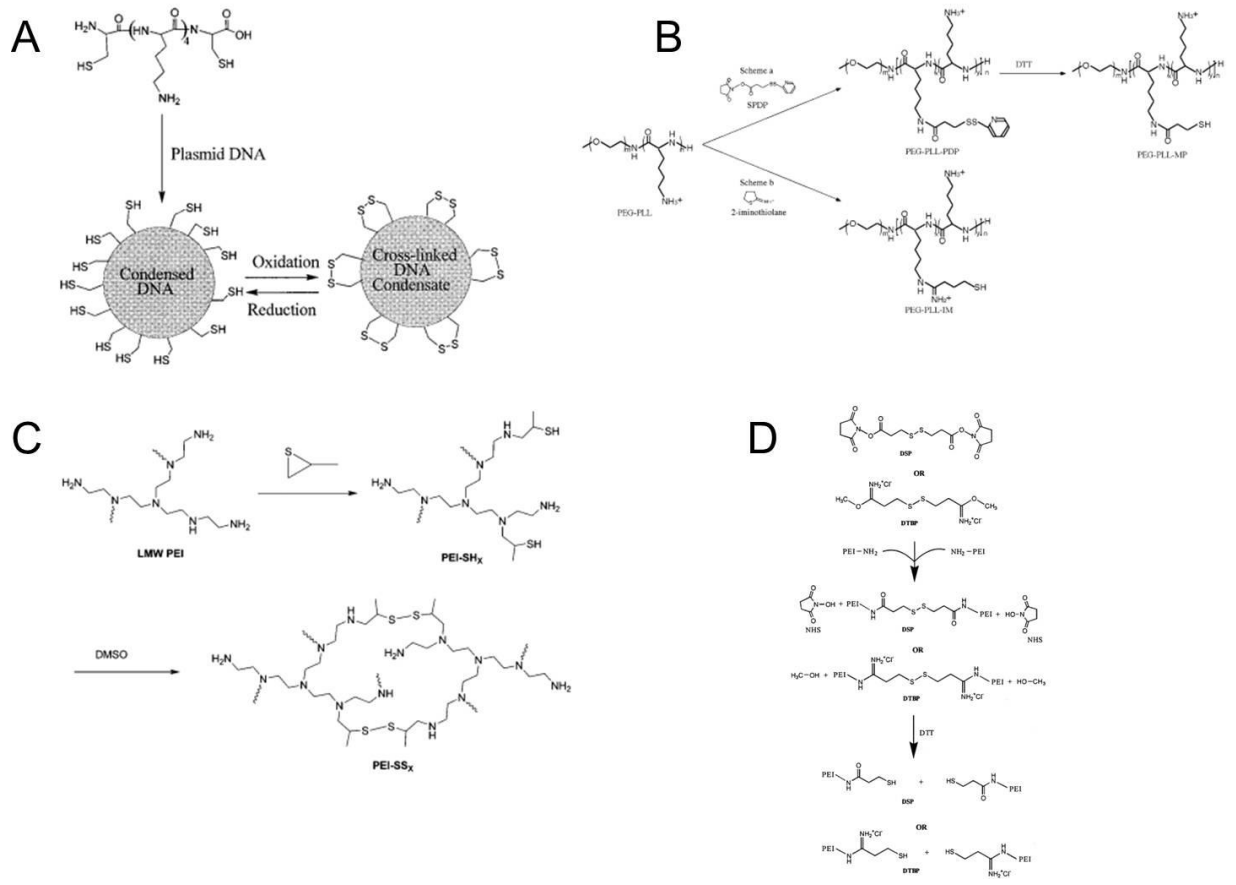

Figure 1.6: Reaction schematics of various disulfide cross-linked polymers: A) McKenzie et al. synthesized a stable peptide DNA condensate by binding poly-l-lysine peptides containing Cys residues with plasmid DNA through the formation of disulfide bond [91], B) Miyata et al. thiolated poly(ethylene glycol)-poly(L-lysine) block copolymer with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or 2-iminothiolane to form a cationic polymer with disulfide cross-linked backbone [92], C) Peng et al. prepared a disulfide cross-linked PEI through the oxidation of a thiolated PEIs with DMSO D) Gosselin et al. disulfide cross-linked PEI with dithiobis(succinimidylpropionate) (DSP) and dimethyl•3,3-dithiobispropionimidate•2HCl [88],

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Chapter 2

Materials and Methods

2.1 Cells and Plasmids

The MDA-MB-231 human breast carcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). The HeLa human cervical carcinoma cell line was a gift from Dr. Sandra McMasters (University of Illinois, Urbana, IL). Cells were cultured according to their ATCC protocols at 37 ^{o}C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM). The growth medium was supplemented with 10% fetal bovine serum (FBS) and 10% penicillin-streptomycin. The 5.3-kilobase expression vector pGL3 (Promega, Madison, WI), coding for firefly luciferase gene driven by the SV40 promoter and enhancer, was purchased from Elim Biopharm (Hayward, CA) and used without further purification.

2.2 PVP(Fe(III)) Catalyst and Degradable Polyethylenimine Syntheses

Polymer supported ferric chloride was synthesized by suspending 1 g of poly(4-vinylpyridine) (PVP) in 10 mL of 0.1 M $FeCl_3$ solution in double-filtered water in a 20-mL scintillation vial, resulting in a clear yellow solution with white powder in suspension. An additional 161 mg (1 mmol) of $FeCl_3$ was added, and the mixture turned into a cloudy reddish brown color. The mixture was stirred with a stir bar at room temperature for 30 minutes, during which the overall mixture color remained the same, and the solid suspension color changed from white to reddish orange. The PVP-immobilized Fe(III) solid was filtered with filter paper under vacuum, washed with double-filtered water, and dried at 60^{o}C overnight. The resulting catalyst was a reddish orange powder that was used without further purification.

Degradable PEI was synthesized by cross-linking 800 Da branched PEI with 1,6-hexanediol diacrylate cross-linker. Briefly, 1 g of 800 Da branched PEI was dissolved in 10 mL of methanol at room temperature in a scintillation vial. Various molar equivalents of PVP(Fe(III)) catalyst to mole of PEI, between 0.05 to 0.15, in addition to an equimolar amount of cross-linker to PEI, were added into the mixture to form a suspension. The mixture was sealed with a screw cap and wrapped in Parafilm and stirred with a stir bar overnight at 60^{o}C. After 24 h, the mixture was transferred into a 15 mL polystyrene tube and centrifuged at 4500 rpm for 10 min to remove the catalyst. Unreacted materials were extracted using approximately 15 mL of petroleum ether. The methanol phase was collected, and methanol was removed by using a rotating evaporator at 67 ^{o}C and 150 rpm for approximately 25 min until only viscous degradable PEI sample was left. Polymer was used without further purification and stored at -80^{o}C.

2.3 Determination of Molecular Weight

Based on the method previously described by von Harpe et al., capillary viscometery was used to determine the molecular weight of the degradable polymer samples. Briefly, polymers were dissolved in 0.5 M $NaNO_3$ to achieve three different solutions with concentrations between 1 to 5 g/L, and the viscosities were measured using a kinematic viscometer (Cannon, State College, PA) at 25 ^{o}C. Due to the high cationic charge of the polymer, the high salt concentration of $NaNO_3$ is needed in order to obtain a linear relationship between polymer concentration and viscosity. Reduced, specific, inherent, and limiting viscosities of the polymer solution were calculated using Equations 1, 2, and 3:

$$\eta_{sp} = (\eta - \eta_0)/\eta_0 \approx t/t_0 \quad \text{..... (1)}$$

$$\eta_{red} = \eta_{sp}/c \quad \text{..... (2)}$$

$$[\eta] = \lim_{c \to 0}(\eta_{red}) \quad \text{..... (3)}$$

where η is the viscosity of the polymer sample in solvent, η_0 is the viscosity of the solvent (determined experimentally), η_{sp} is the specific viscosity of the polymer sample in solvent, t is the time the polymer sample in solvent took to go through the viscometer, t_0 is the time the solvent took to go through the viscometer, η_{red} is the reduced viscosity of the polymer sample in solvent, c is the concentration of polymer in the solvent, and $[\eta]$is the intrinsic viscosity of the polymer sample in solvent.

Plotting $\log([\eta])$ against $\log(MW)$, a linear relationship was constructed using Staudinger-Mark-Houwink relationship with known molecular weight PEI standards (Eqn 4):

$$\log([\eta]) = (0.26)\log(MW) - 2 \quad \text{..... (4)}$$

By measuring the viscosity of each cross-linked polymer sample, one can calculate the molecular weight of the sample by using Eqn 1-4.

2.4 ^{1}H NMR for Cross-linking Density Determination

To determine the degree of polymer cross-linkage, 10 mg of each polymer was dissolved in D_2O and ^{1}H-NMR spectrum was acquired using Varian Unity 400 with a 5-mm probe. The extent of cross-linkage was determined by peak integration at: δ=1.25-1.40 ppm (br m, -$COOCH_2CH_2CH_2$-, 2 H, ester linker), δ=1.40-1.60 ppm (br m, -$COOCH_2CH_2$-, 2 H, ester linker), δ=2.33-2.47 ppm (br m, $CH_2CH_2NHCH_2CH_2COOCH_2$, 2 H, ester linker), δ=2.47-3.3 ppm (br m, CH_2CH_2N, PEI ethylenes), δ=3.5-3.6 ppm (t, ^{3}J=6.6MHz, -$HOCH_2$-, 2 H, hydrolyzed ester linker), and δ=4.1 ppm (m, -$COOCH_2$-, 2H, ester linker). For the cross-linked PEI degradation study, each sample was dissolved in D_2O and incubated at 37 ^{o}C for 0 h, 24 h, 72 h, and 192 h, and the ^{1}H spectrum was acquired using the same apparatus. The fraction of remaining cross-linked

PEI was determined by integrating and comparing the ester peaks at δ=4.1 ppm (m, -COOCH_2, 2H, ester linker) and δ=3.5-3.6 ppm (t, ^{3}J=6.6MHz, -HOCH_2, 2 H, hydrolyzed ester linker).

The cross-link density of the cross-linked PEI was calculated based on the results from elemental analysis of the sample using mass balances, carbon and nitrogen balances in particular (Eqn 5-6):

$$(12)*(a)*(x)+(12)*(b)*(y)=\alpha \quad \text{..... (5)}$$

$$(14)*(c)*(x)+(14)*(d)*(y)=\beta \quad \text{..... (6)}$$

where a is the number of carbon atoms in the PEI monomer, b is the number of carbon atoms in 1,6-hexanediol diacrylate, c is the number of nitrogen atoms in PEI monomer, d is the number of nitrogen atoms in 1,6-hexanediol diacrylate, x is the number of mole of cross-linked PEI monomer, y is the number of mole of cross-linked 1,6-hexanediol diacrylate, α is the weight fraction of carbon in the cross-linked PEI sample, and β is the weight fraction of the nitrogen in the cross-linked PEI sample. With the values of a, b, c, and d determined from the final product structure, x and y can be calculated. The cross-linking density was calculated based on the ratio of x and y, and the assumption that there are an average of 20 PEI monomer units per PEI chain.

2.5 Characterization of Polyplexes

2.5.1 Gel Retardation Studies

One microgram of DNA was diluted into 10 µL of 120 mM NaCl, 16 mM PIPES at pH 7.2. Various amounts of polymer were dissolved in double distilled water and added to the 10 µL DNA/PIPES solution to form the desired polymer and DNA weight–to-weight ratio polyplexes. The polyplexes were incubated at room temperature for 15 min before adding DNA sample

buffer (Biorad). The samples were electrophoresed on a 1% agarose gel for 30 min at 120 V. The gel was visualized with ethidium bromide staining.

2.5.2 Ethidium Bromide Exclusion Assay

One microgram of DNA was diluted into 250 µL of 120 mM NaCl, 16 mM PIPES at pH 7.2. Various amounts of polymer were dissolved in double distilled water and added to the 250 µL DNA/PIPES solution to form the desired polymer and DNA weight–to-weight ratio polyplexes. The polyplexes were incubated at room temperature for 15 min in a 96-well microplate before adding 0.5 µg of 1 µg/µL ethidium bromide into each polyplex sample to reach a final ethidium bromide concentration of 5 µM and incubating at room temperature for another 10 min. Fluorescence was excited at 510 nm and emission detected at 595 nm (Bio Tek, Winooski, VT). The sample fluorescence value was normalized by values for wells containing DNA only, after subtracting background fluorescence measured from wells containing ethidium bromide only. Each measurement was performed in triplicate.

2.5.3 Heparan Sulfate Displacement

Polyplexes with 1 µg DNA were formed based on the optimal polymer/DNA ratio for transfection for each polymer in 15 µL of 120 nM NaCl, 16 mM PIPES at pH 7.2. Polyplexes were incubated at room temperature for 15 min before various amounts of heparan sulfate, dissolved in double-distilled water, were added into each sample to achieve various weight-to-weight ratio between heparan sulfate and DNA. The mixture was incubated at room temperature for another 15 min after which 3 µL of DNA sample buffer was added into each mixture. The final mixture was loaded onto a 1% agarose gel and electrophoresed for 30 min at 120 V. The gel was visualized with ethidium bromide. For the PEI degradation study, each polymer was incubated at 37 ^{o}C for 0 h, 24 h, 72 h, and 192 h. Polyplexes and heparan sulfate displacement assay were prepared as mentioned above.

2.5.4 Dynamic Light Scattering

One microgram of DNA was diluted into 200 µL of 120 mM NaCl, 16 mM PIPES at pH 7.2. Polymer was dissolved in double distilled water. The amount of sample used was determined by the optimal transfection ratio and diluted into 200 µL of 120 mM NaCl, 16 mM PIPES at pH 7.2, and was added to the 200 µL DNA/PIPES solution to form the desired polymer and DNA weight–to-weight ratio polyplexes. The polyplexes were incubated at room temperature for 15 min and double distilled water was added to reach a final sample volume of 2 mL. The polyplexes' size was measured by Brookhaven Instruments Corporation 90 Plus Particle Size Analyzer (Holtsville, NY) before and after 4 h of incubation at 37 ^{o}C. Each measurement was repeated five times.

2.6 In Vitro Transfection

Cells (MDA-MB-231 or HeLa) were cultured in DMEM with 10% fetal bovine serum and 10% penicillin-streptomycin. In a 24-well plate, $8x10^4$ cells/well were seeded in growth medium with serum 24 h before transfection. On the day of the transfection, growth medium was replaced with fresh serum-free DMEM. Before transfection, 50 µL polyplexes in 120 nM NaCl, 16 mM PIPES at pH 7.2 were formed with 0.5 µg pGL3 and various amount of polymer to achieve desired weight-to-weight ratio. The polyplexes were incubated at room temperature for 15 min and added into each well (50 µL polyplexes/well). Four hours post-transfection, DMEM was replaced with fresh growth medium with serum, and incubated for another 20 h. Twenty-four hours post-transfection, luciferase expression was determined using the Promega Luciferase Assay System (Promega), which reports expression levels as relative light units (RLU) as measured on the Lumat L 9507 luminometer (Berthold, GmbH, Germany). Each experiment was performed in quadruplicate. Luciferase expression results (RLUs) were normalized to total cell protein amount using BCA Protein Assay Kit (Thermo Scientific, Rockford, IL)

2.7 Cytotoxicity

The cytotoxicity of polymer on the MDA-MB-231 and HeLa cells were characterized using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega) according to manufacturer's protocol. Briefly, $8x10^3$ cells/well were seeded in 96-well microplate in growth medium containing 10% FBS and incubated at 37 ^oC and 5% CO_2 overnight. Approximately 24 h after seeding, the medium was replaced with serum-free DMEM, and various amount of polymer was added to the cells to achieve a final concentration between 0 and 50 µg/mL. Following a 4 h incubation period at 37 ^oC and 5% CO_2, the medium was replaced with fresh serum-containing medium and incubated for another 24 h, after which 15 µL of the Dye Solution was added into each well, and cells were incubated for another 4 h. After incubation, 100 µL of the Solubilization Solution/Stop Mix was added into each well and incubated for another 1 h. Afterward, the absorbance at 570 nm, with reference wavelength of 650 nm, was measured using Tecan Safire 2 Fluorescence Reader (Tecan, Mannedorf, Switzerland). The absorbance of medium only was subtracted from the viable cell absorbance and normalized to cells with no polymer. Each polymer and polymer concentration were tested six times in each experiment.

2.8 Polyplex Internalization

Cells (MDA-MB-231 or HeLa) were cultured in DMEM with 10% fetal bovine serum (FBS) and 10% penicillin-streptomycin. In a 24-well plate, $8x10^4$ cells/well were seeded in growth medium with serum 24 h before transfection. On the day of the transfection, growth medium was replaced with fresh serum-free DMEM. The intercalating dye YOYO-1 was mixed with pGL3 according to the ratio 25 nL YOYO-1 for every 1 µg DNA (67 bp/YOYO-1). Fifty microliters of polyplexes in 120 nM NaCl, 16 mM PIPES at pH 7.2 were formed with 0.5 µg pGL3/YOYO-1 and various amount of polymer to achieve desired weight-to-weight ratio. Two hours post-transfection, the cells were washed with 0.001% SDS in PBS to remove surface-bound complexes. One hundred microliters of non-phenol trypsin was added into each well and

incubated for 10 min before adding 350 µL PBS and 50 µL FBS. The cells were collected and stored on ice before measurement. FACS measurements were performed on a BD LSR II Flow Cytometer System (BD, Franklin Lakes, NJ). To determine uptake, median peak fluorescence of polyplexes was recorded and normalized with pGL3/YOYO-1 only samples' median peak fluorescence. Each measurement was performed in triplicate.

Chapter 3

Results

3.1 Biodegradable Polyethylenimine Characterization

3.1.1 Synthesis and Characterization of Biodegradable PEI

Based on the synthesis described in Materials and Methods, three biodegradable PEI samples were synthesized by cross-linking equal moles of 800 Da PEI with 1,6-hexanediol diacrylate, and catalyzed with 0.1, 0.15, and 0.2 molar equivalent of PVP(Fe(III)) catalyst per mole of PEI (**Figure 3.1**). The molecular weights of the biodegradable PEI (D.PEI) samples were 48 kDa, 6.2 kDa, and 1.2 kDa, determined using capillary viscometry (**Table 3.1**) [1]. Compared to the degradable PEIs generated by the non-catalyzed reaction reported by Forrest et al., 14 kDa and 30 kDa D.PEIs [2], this alternative synthesis scheme provides a wider range of D.PEI molecular weights for characterization and gene delivery studies.

The structure and degradation of polymeric gene delivery vectors are important to their impact on safe, efficient gene transfer. Non-degradable PEI may accumulate inside the cell, increasing toxicity, and leading to reduced transfection efficiency. To investigate the structure and degradation of D.PEI samples, we incubated samples at 37 ^{o}C in D_2O for various amounts of time and obtained their ^{1}H NMR spectra. The ^{1}H spectra confirmed the synthesis of ester linkages between PEI and 1,6-hexanediol diacrylate (**Figure 3.2**). To calculate the extent of D.PEI degradation, the relative number of methylene protons in the ester bond and in the hydroxyl group was determined by integrating the corresponding peaks in the spectra (**Figure 3.3 and 3.4**) [6]. The degradation of D.PEIs was the most rapid in the first 24 h and eventually

slowed. Both 48 kDa and 1.2 kDa D.PEIs had about 50% of their initial ester bonds remaining after 24 h of incubation, while 6.2 kDa D.PEI reached its half-life after 72 h incubation.

Another D.PEI characteristic of interest is the degree of cross-linking. Highly cross-linked, low molecular weight PEI is expected to behave similarly to its high molecular weight non-cross-linked counterpart, including improved DNA binding and transfection efficiency, due to similar molecular weights. By utilizing elemental analysis, we approximated the cross-linking density of each D.PEI sample through mass balances (**Table 3.2**). As expected, the higher molecular weight D.PEI possessed higher degree of cross-linking, and vice versa: 48 kDa, 6.2 kDa, and 1.2 kDa D.PEIs have cross-linking density, defined as mole percent of nitrogen atoms in PEI attached to 1,6-hexanediol diacrylate, of 16.9%, 15.1%, and 14.1%, respectively. In addition to cross-linking density, it is also important to understand the cross-linking structure of the D.PEIs. To determine whether the samples have linear or branched structure, we need to calculate the number of PEI chains cross-linked with 1,6-hexanediol diacrylate (**Table 3.2**) [3,7]. The results show that D.PEI samples with higher cross-link density have more diacrylates attached to a PEI chain compared to the lower cross-linked counterpart. More importantly, all three D.PEIs show that, on average, each sample has more than two diacrylates linked with a single PEI chain. This finding implies that the D.PEI samples possibly consist of branched structure. In particular, the 48 kDa D.PEI has more than three diacrylates attached to a PEI chain, which indicates a high level of branching. Due to the loss of flexibility and steric hindrances as a result of this branching, the D.PEIs tend not to condense DNA as efficiently as their unmodified 25 kDa counterpart, a trend which will be discussed below in greater detail.

3.1.2 DNA Migration Inhibition

One of the important characteristics of a good non-viral gene delivery vehicle is its ability to condense DNA efficiently, in order to be taken up by the cells. To measure the capability of the

polymer to condense DNA, one can electrophorese polyplexes formed at various polymer:DNA weight-to-weight ratio in agarose gel to observe the necessary amount of polymer needed to completely inhibit the DNA's migration (**Figure 3.5**). Unmodified 25 kDa PEI needed 0.2 μg polymer/μg DNA to completely retard the DNA. The D.PEI samples were able to retard DNA migration at 0.5 μg polymer/μg DNA ratio. Upon closer examination, one can see 48 kDa D.PEI prevented migration of most of the DNA with 0.3 μg of polymer, while 0.4 μg of polymer was needed for 6.2 kDa D.PEI and 1.2 kDa D.PEI. These results indicate unmodified 25 kDa PEI condensed DNA much more efficiently than the three D.PEI samples, even though we expected that cross-linking 800 Da PEI would result in polymer that behaved more like its higher molecular weight counterpart. This phenomenon can be attributed to a steric effect where some of the primary and secondary amines are covalently bonded with the cross-linker, and as a result, the amine groups are either shielded from the DNA or lost their flexibility to bind to the DNA, compared to the amines in unmodified PEI.

3.1.3 Polyplex Sizing via Dynamic Light Scattering

As mentioned earlier, the size of the polyplex directly affects its ability to be endocytosed by the cells. It is critical that the cationic polymer is able to condense DNA and form nanoscale complexes. To determine the size of the D.PEI complexes, dynamic light scattering technique was used to measure the polyplexes' effective diameters immediately after polyplex formation at each polymers' optimal transfection ratio, and again after 4 hours of incubation at 37 ^{o}C. One would expect the polyplex size to increase after incubation due to polyplex aggregation. . It is indeed the case, as shown in **Table 3.3**. Unmodified 25 kDa PEI polyplexes have the smallest effective diameter both before and after incubation, despite these polyplexes consisting of 7.5-fold to12.5-fold less polymer than the three D.PEI polyplexes. This result shows that unmodified 25 kDa PEI has higher DNA condensation strength than the D.PEIs, which indicates the

presence of higher cationic surface charges. For the D.PEIs, the polyplexes' diameters are inversely related to the molecular weights of the polymers before incubation. 1.2 kDa D.PEI polyplexes have the largest effective diameter, even though there was ~1.6-fold more polymer present, confirming that higher molecular weight PEI can condense DNA better than its lower molecular weight counterpart. The trend is not as clear after 4 h of incubation but one can still see the inverse relationship between molecular weight and polyplex size among the D.PEI samples.

3.1.4 DNA Condensation Strength of PEI

Besides the ability of condensing DNA, a good polymeric gene delivery vector must exhibit tight binding to the DNA to avoid early DNA release, which would reduce the efficiency of gene delivery. One way to measure the tightness of DNA binding is to measure the polymer's ability to exclude ethidium bromide, an intercalating dye that would attach to DNA and fluoresce, as an indicator of the "tightness" of the DNA condensation. To quantify the degree of condensation between D.PEIs and DNA, ethidium bromide fluorescence was measured by mixing ethidium bromide with various polymer and DNA ratio polyplexes (**Figure 3.6**). Unmodified 25 kDa PEI condensed DNA tightly at polymer:DNA weight-to-weight ratio 0.5:1 showing only 1% normalized fluorescence. The D.PEI samples required more polymers, 0.5 μg, 1 μg, and 1 μg of polymer:DNA ratio for 48 kDa, 6.2 kDa, and 1.2 kDa D.PEI, respectively, to achieve minimum normalized fluorescence and similar DNA binding "tightness" as unmodified 25 kDa PEI. This indicates that although all PEI samples are able to tightly condense DNA with only a small amount of polymers, D.PEIs seem to form "looser" polyplexes than unmodified PEI. The normalized fluorescence of all PEI samples gradually increased and reached a plateau as polymer:DNA ratio increases. This is unexpected and is likely an artifact contributed by the

prolonged incubation time during sample preparation between polyplexes and ethidium bromide.

3.1.5 DNA Competitive Displacement

Upon entering the cells, the DNA inside the polyplexes must be released from the polyplexes in order for transcription to occur. One way to measure the polymer's DNA condensation, in respect to DNA release, is to displace the DNA with anionic proteoglycan like heparan sulfate. Heparan sulfate (HS) competitively displaces DNA from cationic vectors, and the amount of HS needed to completely replace DNA from polyplexes indicates the strength of the polymer's DNA binding [4-5]. To determine the relative DNA binding strength of the D.PEIs before and after degradation (0 to 192 hours incubation at 37 ^{o}C), along with unmodified 25 kDa and 800 Da PEIs, various amount of HS was mixed into polyplexes prepared at 2:1 polymer/DNA weight-to-weight ratio and electrophoresed in agarose gel (**Figure 3.7**). For unmodified PEIs, 25 kDa PEI and 800 Da PEI needed about 18 μg HS/μg DNA and 12 μg HS/μg DNA to displace the DNA, respectively, as expected. All the D.PEI samples needed less HS compared to unmodified 25 kDa PEI, about 12 μg HS/ μg DNA, to dissociate the DNA. This indicates it is easier to unpackage DNA from the D.PEIs than the unmodified PEI, which might explain the more efficient gene transfer of D.PEIs. Approximately the same amount of HS is needed for all D.PEI samples and for the 800 Da PEI, indicating that molecular weight of the D.PEI is not the only factor that controls DNA binding. This explains why even the 48 kDa D.PEI sample needs less HS to displace DNA compared to the 25 kDa control. 25 kDa PEI represents a long, continuous network of cationic PEI, whereas D.PEI is a lowly cross-linked network of shorter (800 Da) PEI pieces with cross-linker covalently attached to its primary and secondary amines in between. As result, due to the steric effect and loss of flexibility discussed previously, DNA will attach more strongly to the higher overall positive charge of 25 kDa PEI than even the highest molecular

weight of cross-linked D.PEI. In the tested incubation period, there is no significant change in the amount of HS needed to release DNA from the polyplexes for all D.PEI samples. This is expected since D.PEIs and 800 Da PEI released DNA with the same amount of HS present, as a result, D.PEI degradation should not affect the competitive release of DNA. This could be due to the relatively low cross-linking density of the D.PEIs, where the DNA binding of the unmodified 800 Da PEI, the starting material of the D.PEI, might overwhelm the effect of the reduced DNA binding strength from cross-link degradation.

3.2 Biodegradable Polyethylenimine Gene Delivery

3.2.1 In Vitro Transfection

Transfection efficiency of unmodified and cross-linked PEIs were studied in HeLa and MDA-MB-231 cell lines (**Figure 3.8** and **Figure 3.9**). In HeLa cells, at their respective optimal polymer:DNA weight-to-weight ratio, both 48 kDa and 6.2 kDa D.PEIs transfected about 5–fold more efficiently than unmodified 25 kDa PEI. Though not as efficient as the other two D.PEIs, the transfection efficiency of 1.2 kDa D.PEI is about 3-fold higher than that of unmodified PEI. In MDA-MB-231 cells, D.PEIs also mediated greater transgene expression than 25 kDa PEI, but not as significantly as with HeLa cells. At their optimal polymer:DNA ratio, 48 kDa and 6.2 kDa D.PEIs's transfection efficiency was about 1.6–fold higher than unmodified PEI, while 1.2 kDa D.PEI shows similar transfection efficiency as unmodified PEI. The optimal transfection polymer:DNA ratio was 2:1 for unmodified 25 kDa PEI in both HeLa and MDA-MB-231 cells. However, the D.PEIs' optimal transfection polymer:DNA ratios were different between the two cell lines. In HeLa cells, the optimal polymer:DNA ratios were 10:1, 10:1, and 25:1 for 48 kDa, 6.2 kDa, and 1.2 kDa D.PEIs, respectively. In MDA-MB-231 cells, the optimal ratio for 48 kDa and 6.2 kDa D.PEIs was 15:1, while 1.2 kDa D.PEI's optimal ratio was 20:1. For all the D.PEI samples, the increased amount of polymers needed to achieve the optimal polymer:DNA ratio is

likely due to the weaker DNA condensation and “looser” DNA binding compared to unmodified PEI. As a result, to compensate, more polymers are needed to improve transfection efficiency.

3.2.2 Cytotoxicity

For any therapeutic treatment, safety is of the utmost importance. Therefore, it is important to choose materials that show minimal toxicity as a gene delivery vehicle. Studies have shown that higher molecular weight PEI is significantly more cytotoxic than its lower molecular weight counterparts [2-3]. In order for the D.PEIs to be considered as potential vectors for gene delivery, they need to demonstrate lower cytotoxicity than unmodified 25 kDa PEI. To measure the polymers’ cytotoxicity, the MTT assay was used to measure viability of HeLa and MDA-MB-231 cells when they were exposed to various amounts of polymers (**Figure 3.10** and **Figure 3.11**). Unmodified 25 kDa PEI displayed highest cytotoxicity and reduced cell viability to 10% at polymer concentrations of 15 μg polymer/mL and 30 μg polymer/mL in HeLa and MDA-MB-231 cells, respectively. All D.PEIs show significantly lower cytotoxicity in both cell lines compared to unmodified PEI. 48 kDa D.PEI is the most toxic of the three D.PEI samples; at 50 μg polymer/mL, it yielded 40% and 80% cell viability in HeLa and MDA-MB-231 cells. 1.2 kDa D.PEI is the least toxic D.PEI sample and showed minimal cytotoxicity in both HeLa and MDA-MB-231 cells, 110% and 95% cell viability at 50 μg polymer/mL, respectively. At the same concentration, 6.2 kDa D.PEI displayed 50% and 100% viability in the two cell lines. Regardless of polymer samples, MDA-MB-231 cells consistently show lower cell viability than HeLa cells, indicating that PEI is generally more toxic to MDA-MB-231 cells than to HeLa cells.

3.2.3 Cellular Uptake

Cationic polyplexes need to go through many cellular processes in order for transfection to succeed, the first of which is endocytosis. As discussed earlier, endocytosis is the process cells

use to uptake the polyplexes into the cytosol. It is generally a non-specific process unless there are targeting ligands attached to the polyplexes. By using fluorescence-activated cell sorting (FACS), we can measure the uptake of the fluorescently labeled polyplexes through non-specific endocytosis and determine any existing relationship between transfection and cellular uptake of the polyplexes (**Figure 3.12** and **Figure 3.13**). Overall, MDA-MB-231 cells endocytosed 2- to 3-fold more polyplexes than HeLa cells for all polyplexes at the tested polymer:DNA weight-to-weight ratios. For unmodified 25 kDa PEI, approximately the same amount was endocytosed across various polymer:DNA ratios in both HeLa and MDA-MB-231 cells. HeLa cells displayed similar uptake of D.PEI and unmodified PEI polyplexes, except at the highest tested polymer:DNA ratio, 25:1, where the cells endocytosed slightly more 48 kDa D.PEI polyplexes than unmodified PEI polyplexes. Unlike HeLa cells, MDA-MB-231 cells endocytosed more D.PEI polyplexes than unmodified PEI polyplexes in the tested polymer:DNA ratios, except at the lower polymer: DNA ratios (2:1 and 5:1). At their respective optimal uptake polymer:DNA ratios, 48 kDa, 6.2 kDa, and 1.2 kDa D.PEI polyplexes show 3-, 7-, and 4-fold higher cellular uptake than unmodified PEI polyplexes.

3.3 Tables and Figures

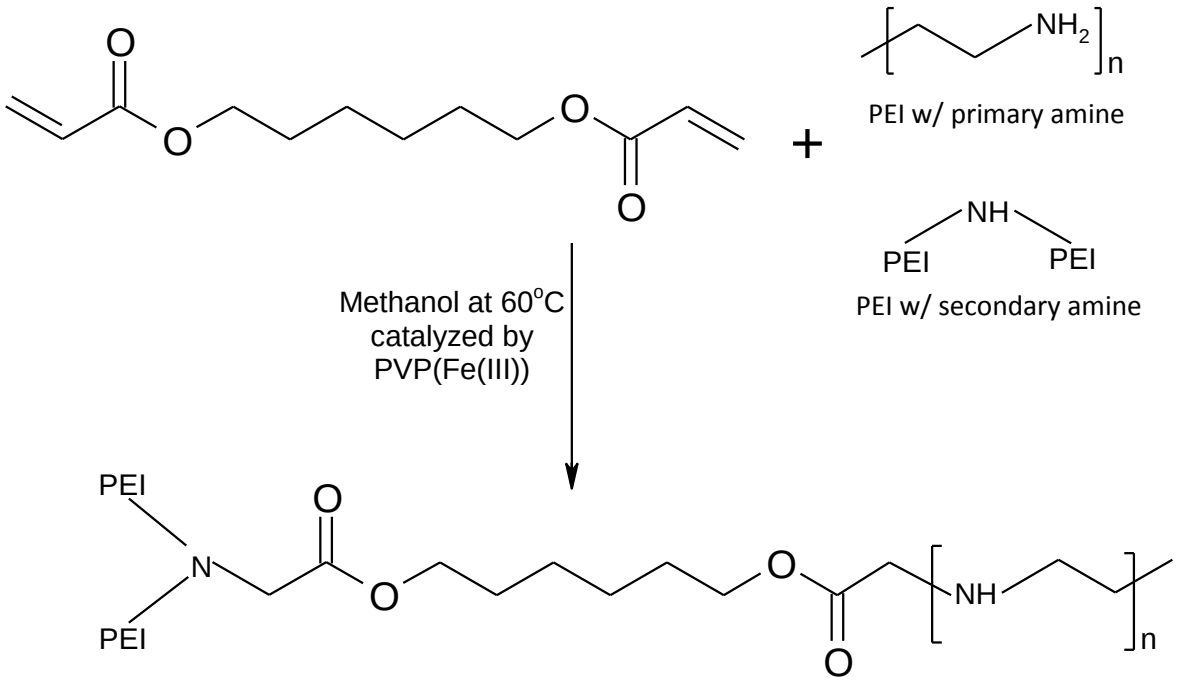

Figure 3.1: Synthesis of biodegradable PEI derivatives. 800 Da PEI is cross-linked with 1,6-hexanediol diacrylate in methanol overnight at 60°C, catalyzed by PVP(Fe(III)) heterogenous catalyst. The diacrylate groups can react with primary and secondary amines of the PEI.

Table 3.1: Polymer concentrations, viscosity measurements, reduced viscosities, and molecular weights of biodegradable PEIs

Molar Ratio of Catalyst to PEI	Concentration (mg/mL)	Average Time (s)	SD	Reduced Viscosity (dL/g)	Molecular Weight (kDa)
	2	240.79	0.33		
0.2	4	247.44	0.44	0.0968	6.2
	6	250.00	0.49		
	2	232.83	13.59		
0.15	4	243.71	0.17	0.0636	1.2
	7	250.75	0.34		
	2	243.58	0.45		
0.1	4	246.63	0.21	0.1651	48
	7	251.59	0.12		

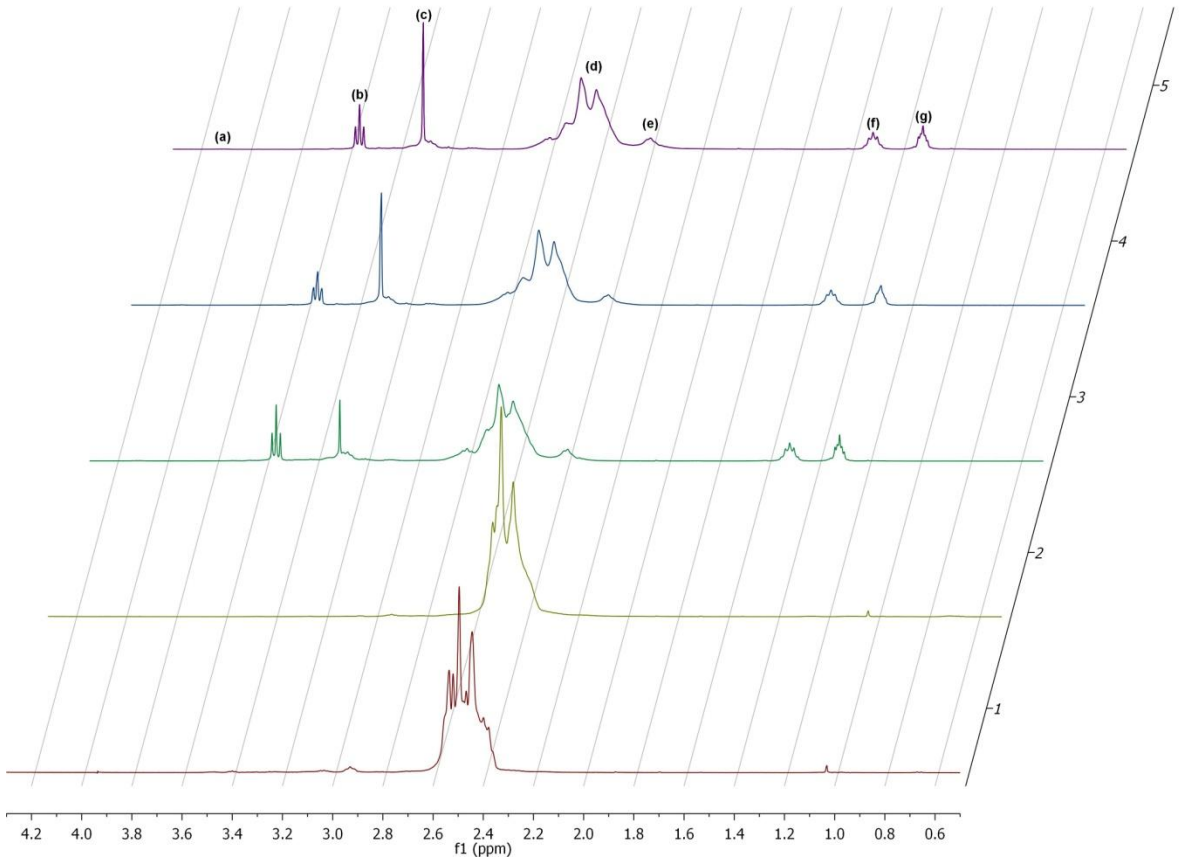

Figure 3.2: NMR spectra of unmodified PEIs and D.PEIs. 1) Unmodified 800 Da PEI, 2) unmodified 25 kDa PEI, 3) 48 kDa D.PEI, 4) 6.2 kDa D.PEI, and 5) 1.2 kDa D.PEI. D.PEI spectrum peak labels: (a) δ=4.1 ppm, -COOCH_2, ester linker; (b) δ=3.5-3.6 ppm, -HOCH_2, hydrolyzed ester linker; (c) δ=3.34ppm, methanol residual; (d) δ=2.47-3.3 ppm, CH_2CH_2N, PEI ethylenes; (e) δ=2.33-2.47 ppm, $CH_2CH_2NHCH_2CH_2COOCH_2$, ester linker; (f) δ=1.40-1.60 ppm, -COOC$H_2$$CH_2$, ester linker; and (g) δ=1.25-1.40 ppm, -$COOCH_2CH_2CH_2$, ester linker.

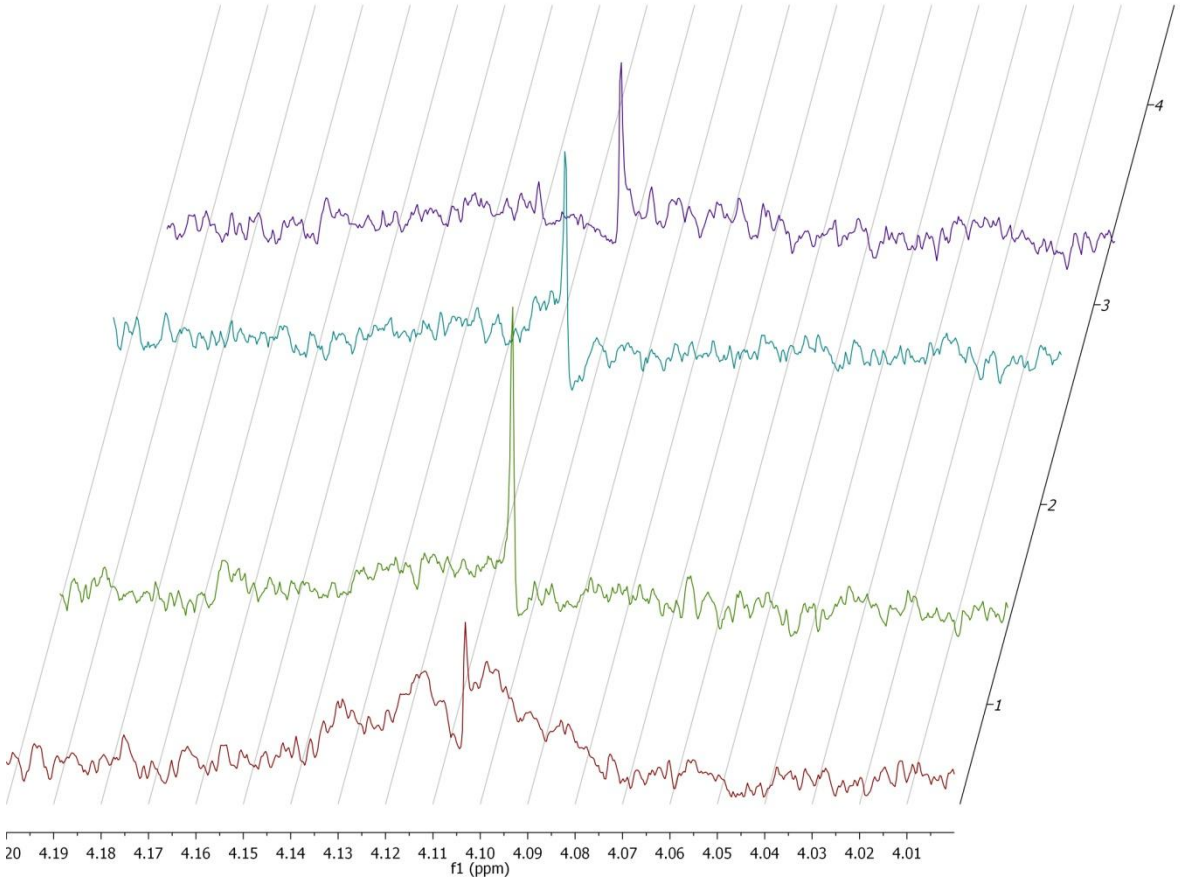

Figure 3.3: NMR spectra of 48 kDa D.PEI showing the change in peak intensity for unhydrolzed ester linker (δ=4.1 ppm) in D_2O at 37^{o}C for various incbuation periods: 1) 0 hour, 2) 24 hours, 3) 72 hours, and 4) 192 hours.

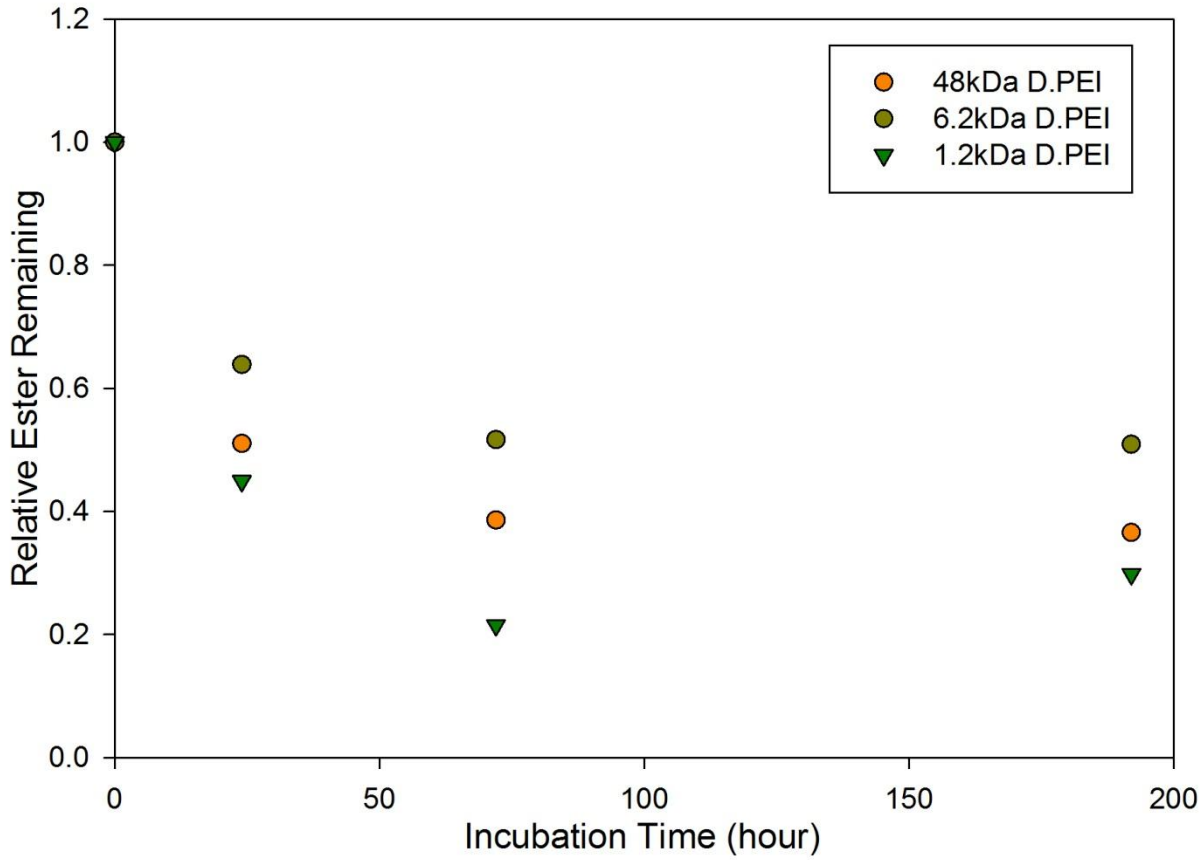

Figure 3.4: Degradation of D.PEIs in D_2O at 37°C for various incbuation periods. Samples were measured with 400 MHz ^1H NMR. Degradation was calculated based on the integrals of the ester linker and hydrolyzed ester linker peaks as described in Chapter 2.

Table 3.2: Elemental analysis results, calculated % cross-linking and # diacrylate cross-linked with a PEI chain for each D.PEI sample

D.PEI Samples	Weight % Carbon	Weight % Nitrogen	Weight % Hydrogen	% Cross-linking	# Cross-linker attached to a PEI chain
48 kDa	46.85	19.96	10.46	16.9	3.14
6.2 kDa	49.07	20.26	10.19	15.1	2.81
1.2 kDa	50.65	20.46	10.50	14.1	2.62

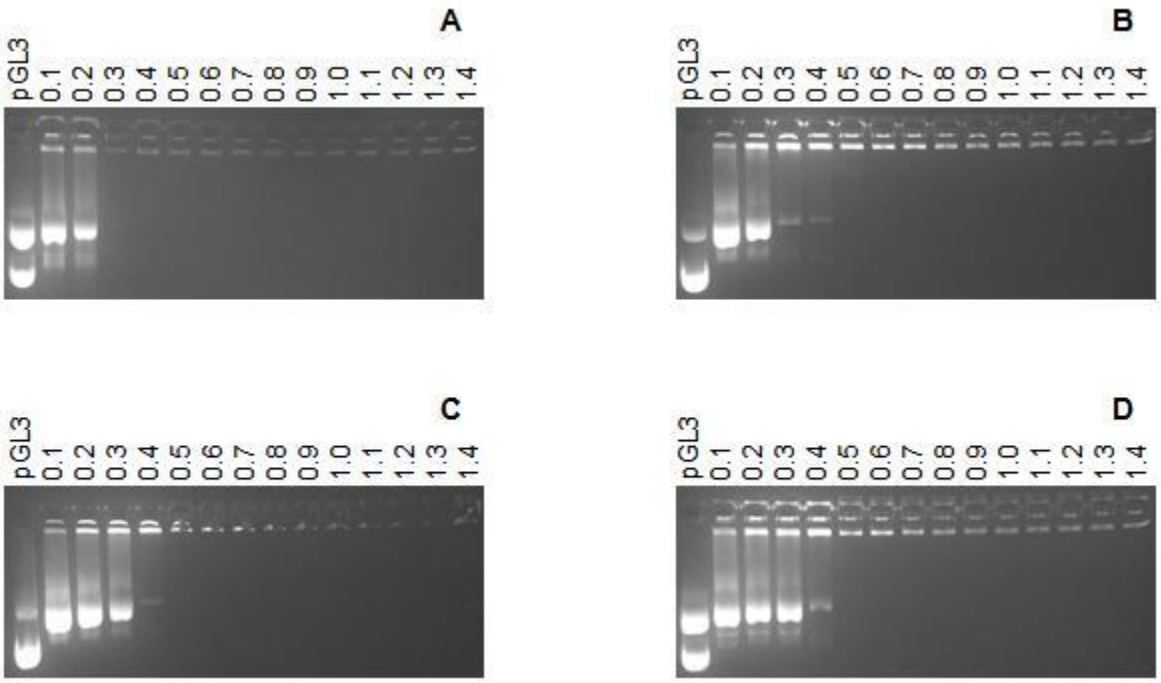

Figure 3.5: Gel retardation study of polymer/DNA polyplexes. A) Unmodified 25 kDa PEI, B) 48 kDa D.PEI, C) 6.2 kDa D.PEI, D) 1.2 kDa D.PEI. The polymer to DNA weight-to-weight ratio for each polyplex formation is listed above the corresponding lane

Table 3.3: The effective diameters of unmodified 25 kDa PEI and three D.PEIs' polyplexes before and after 4 hours incubation at 37^{o}C, at their respective optimal transfection ratio

Polymer	**Polymer:DNA (w/w)**	**Effective Diameter (nm)** *(0 hour incubation)*	**SD**	**Effective Diameter (nm)** *(4 Hours Incubation)*	**SD**
25 kDa Unmodified PEI	2 : 1	293.2	4.1	457.3	16.8
48 kDa D.PEI	15 : 1	321.8	7.8	483.5	8.4
6.2 kDa D.PEI	15 : 1	405.2	5.2	481.5	19.0
1.2 kDa D.PEI	25 : 1	440.4	3.4	551.5	12.0

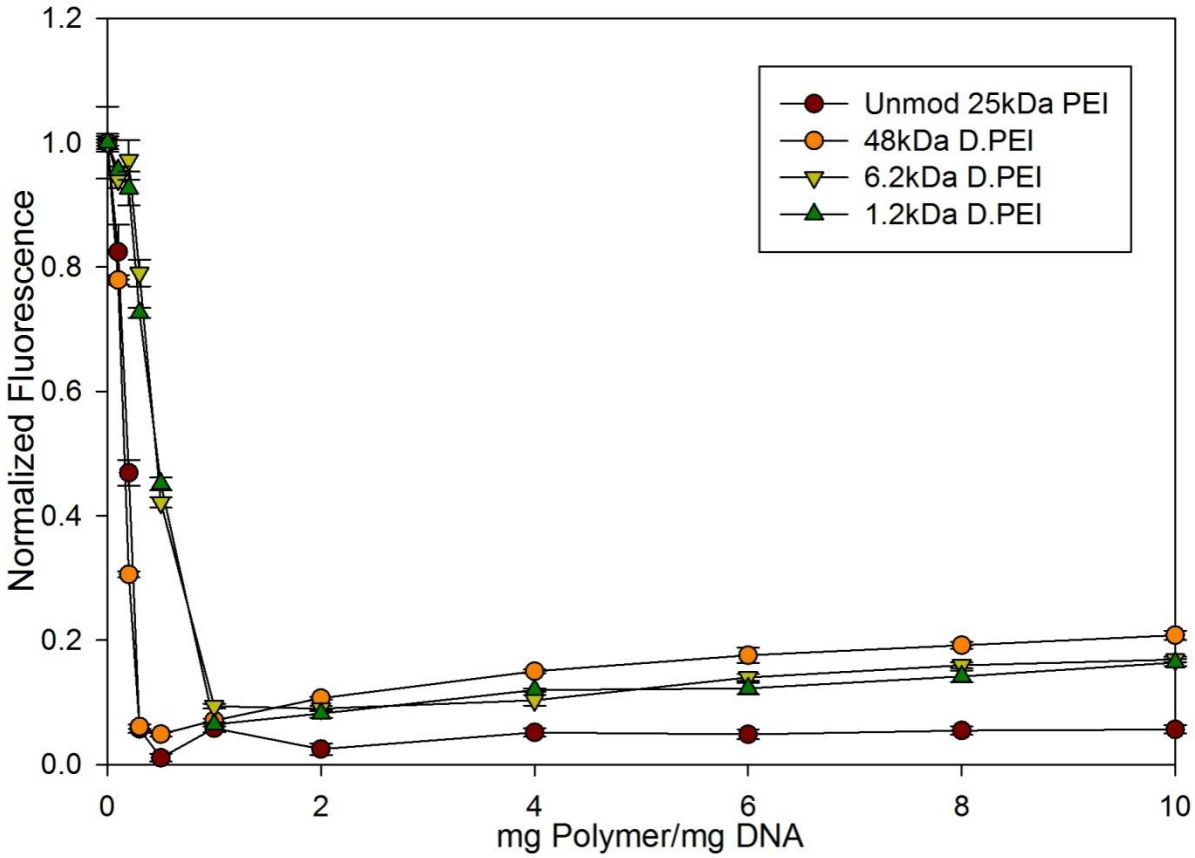

Figure 3.6: Ethidium bromide exclusion assay. Polyplexes were formed with unmodified 25 kDa PEI and D.PEIs with DNA at various weight-to-weight ratios in the presence of ethidium bromide. Normalized fluorescence was calculated by $(F-F_0)/(F_{DNA}-F_0)$. F, fluorescence of polyplexes and ethidium bromide; F_0, fluorescence of ethidium bromide only; F_{DNA}, fluorescence of DNA and ethidium bromide (N=3, error bars represent standard deviation)

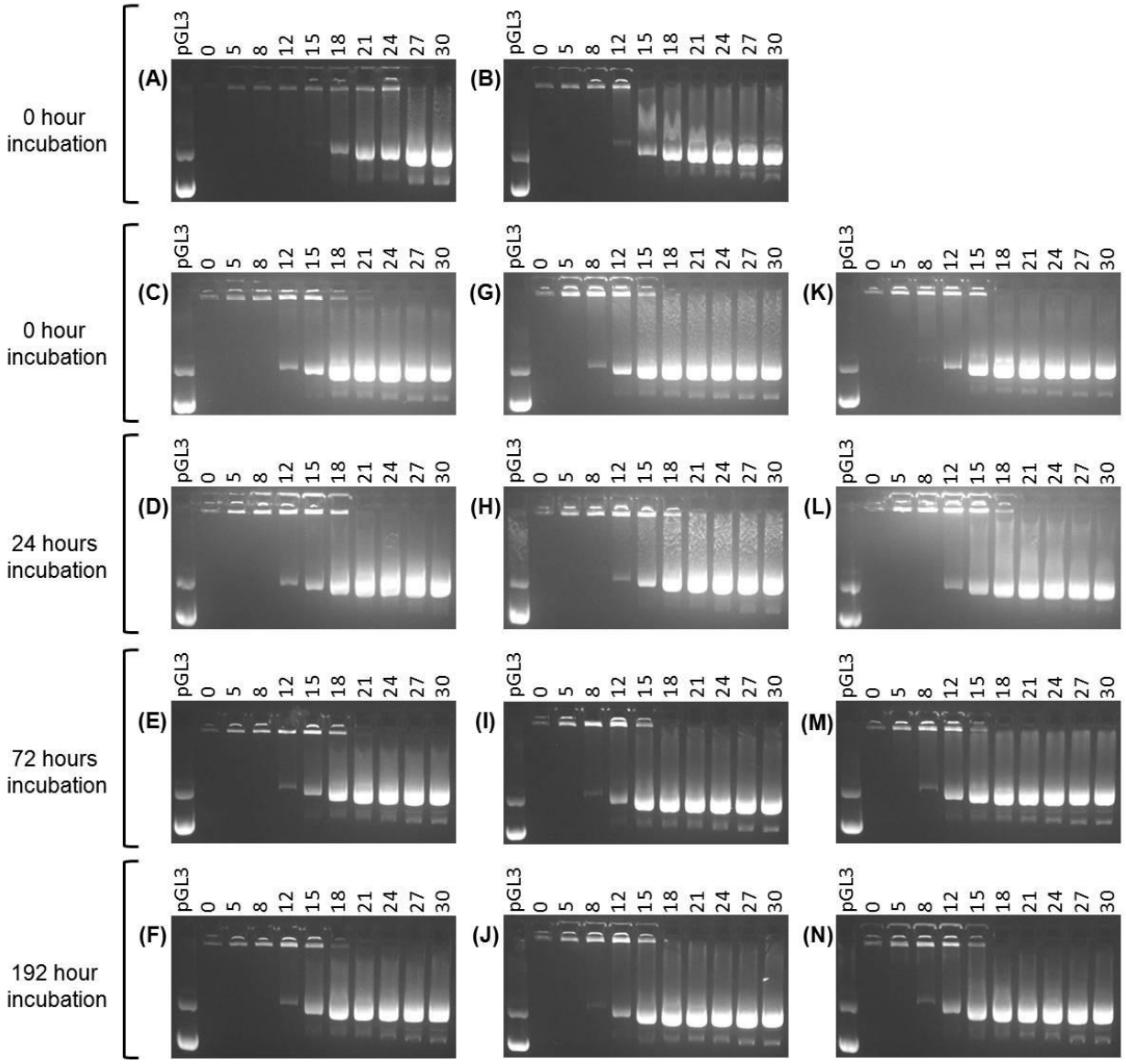

Figure 3.7: Heparan sulfate displacement assay of polymer/DNA polyplexes at 2:1 weight-to-weight ratio. D.PEIs were incubated at 37 ^{o}C for 0, 24, 72, and 192 hours before complexed with DNA. (A) unmodified 25 kDa PEI, (B) unmodified 800 Da PEI, (C-F) 48 kDa D.PEI, (G-J) 6.2 kDa D.PEI, (K-N) 1.2 kDa D.PEI. The polymer to DNA weight-to-weight ratio for each polyplex formation is listed above the corresponding lane

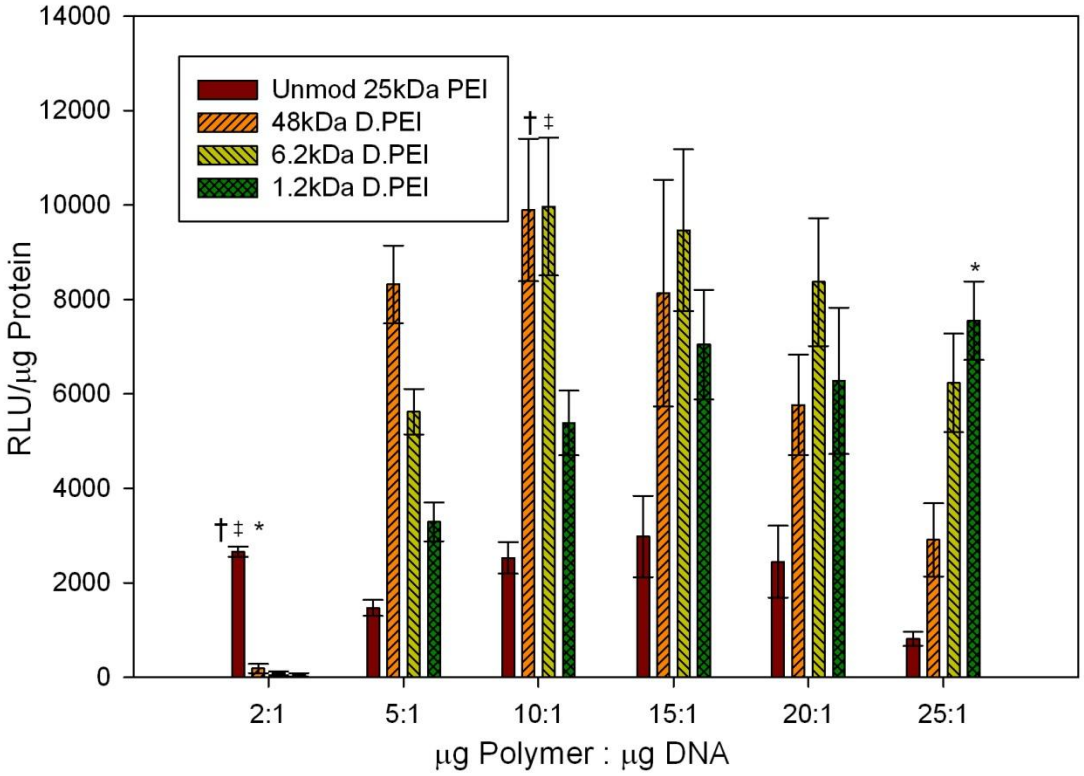

Figure 3.8: In vitro transfection of HeLa cells with polyplexes formed with pGL3 and unmodified 25 kDa PEI or D.PEIs at various polymer:DNA weight-to-weight ratios (t-test: †, $p<0.01$; ‡, $p<0.01$; *, $p<0.01$). Luciferase expression in the cell lysates is reported as relative light unit (RLU) normalized by the total amount of protein the cell lysates (N=4, error bars represent standard deviation).

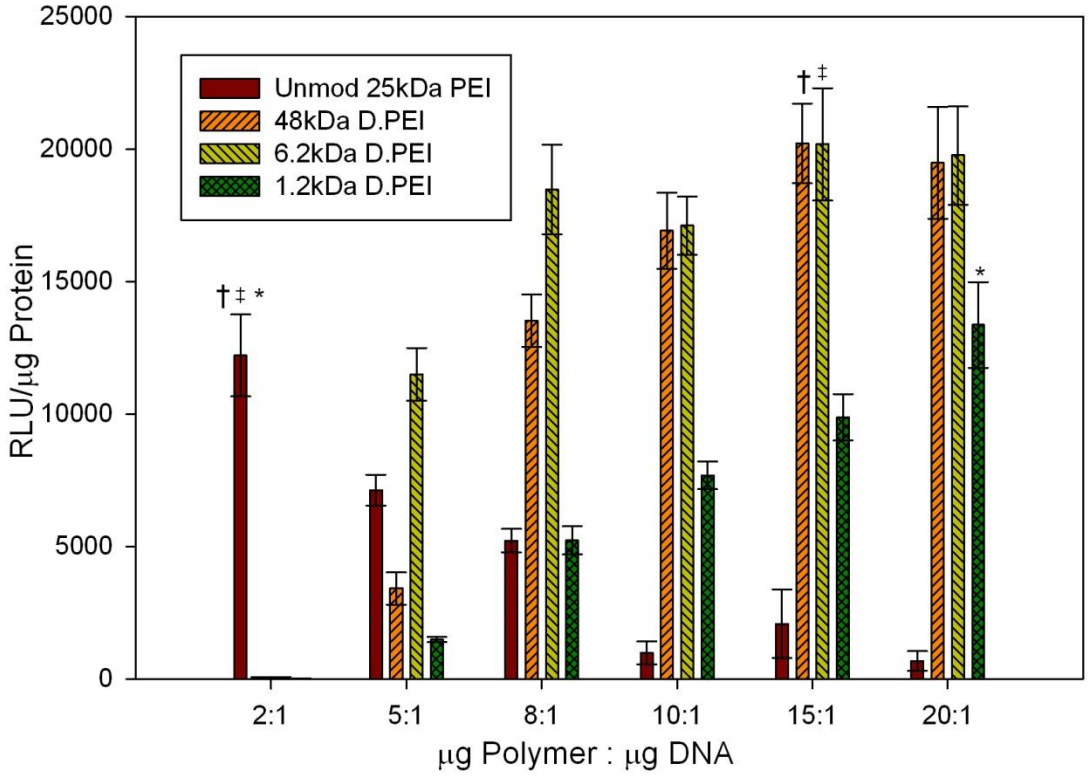

Figure 3.9: In vitro transfection of MDA-MB-231 cells with polyplexes formed with pGL3 and unmodified 25 kDa PEI or D.PEIs at various polymer:DNA weight-to-weight ratios (t-test: †, $p<0.01$; ‡, $p<0.01$; *, $p=0.16$). Luciferase expression in the cell lysates is reported as relative light unit (RLU) normalized by the total amount of protein the cell lysates (N=4, error bars represent standard deviation).

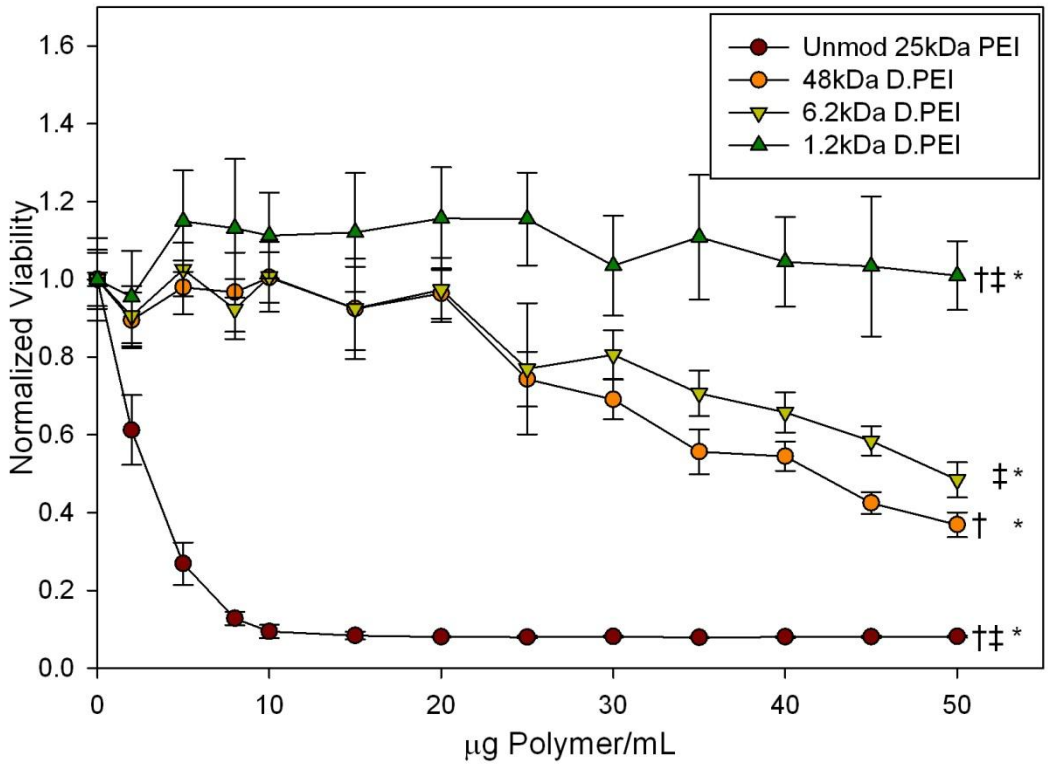

Figure 3.10: Cytotoxicity of biodegradable PEI derivatives reported as normalized metabolic activity in HeLa cell line in the presence of varying amounts of unmodified 25 kDa PEI and D.PEIs (ANOVA: †, 48 kDa D.PEI, $p<0.05$; ‡, 6.2 kDa D.PEI, $p<0.05$; *, 1.2 kDa D.PEI, $p<0.05$). Metabolic activity was normalized to control with no polymer present (N=6, error bars represent standard deviation).

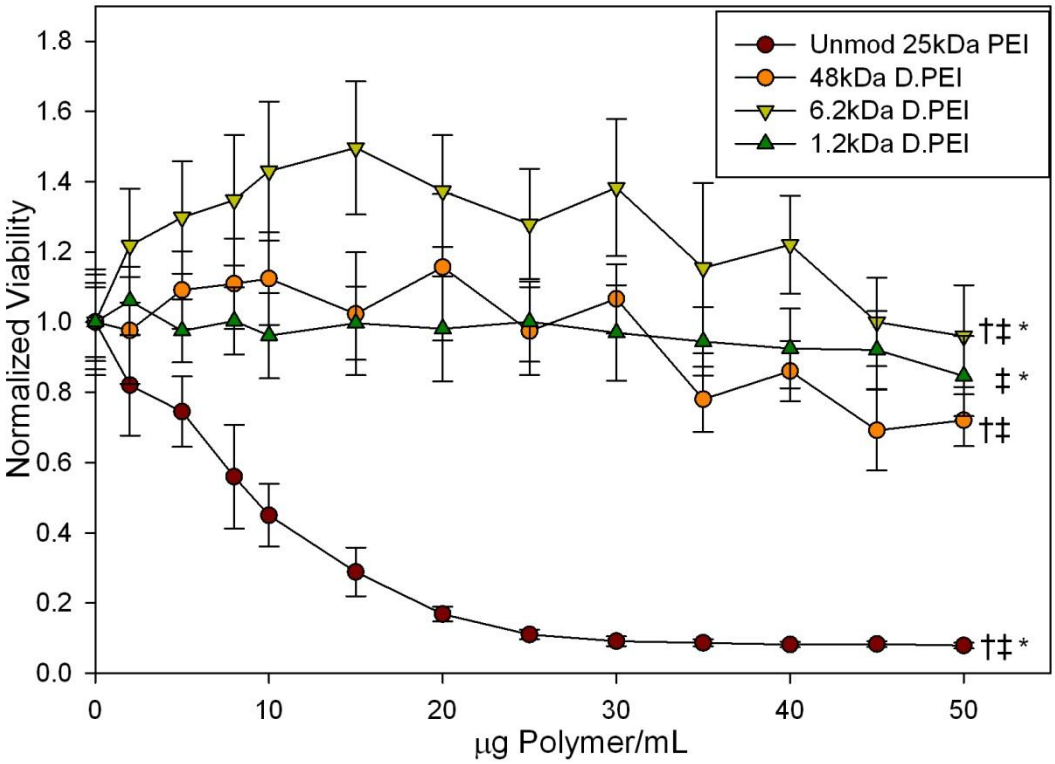

Figure 3.11: Cytotoxicity of biodegradable PEI derivatives reported as normalized metabolic activity in MDA-MB-231 cell line in the presence of varying amounts of unmodified 25 kDa PEI and D,PEIs (ANOVA: †, 48 kDa D.PEI, $p<0.05$; ‡, 6.2 kDa D.PEI, $p<0.05$; *, 1.2 kDa D.PEI, $p<0.05$). Metabolic activity was normalized to control with no polymer presence (N=6, error bars represent standard deviation).

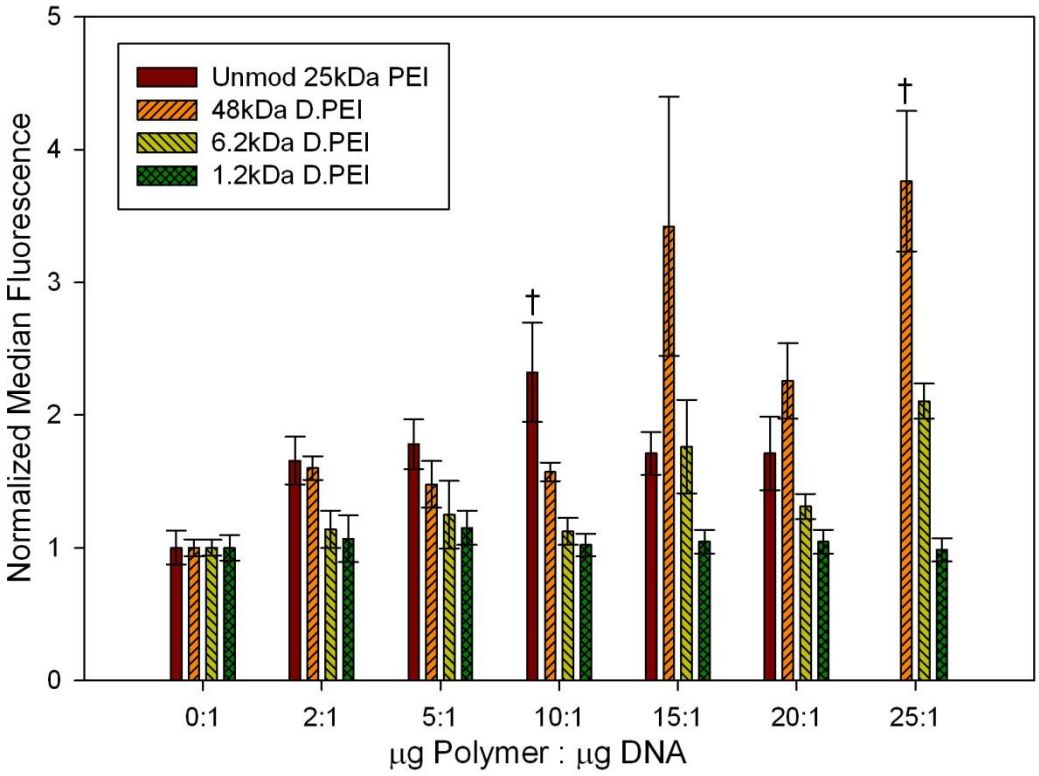

Figure 3.12: Cellular uptake of fluorescently labeled polyplexes with YOYO-1 intercalated pGL3 in HeLa cells (t-test: †, $p<0.01$). Results are reported as median fluorescence normalized by control with no polymer present using FACS (N=3, error bars represent standard deviation).

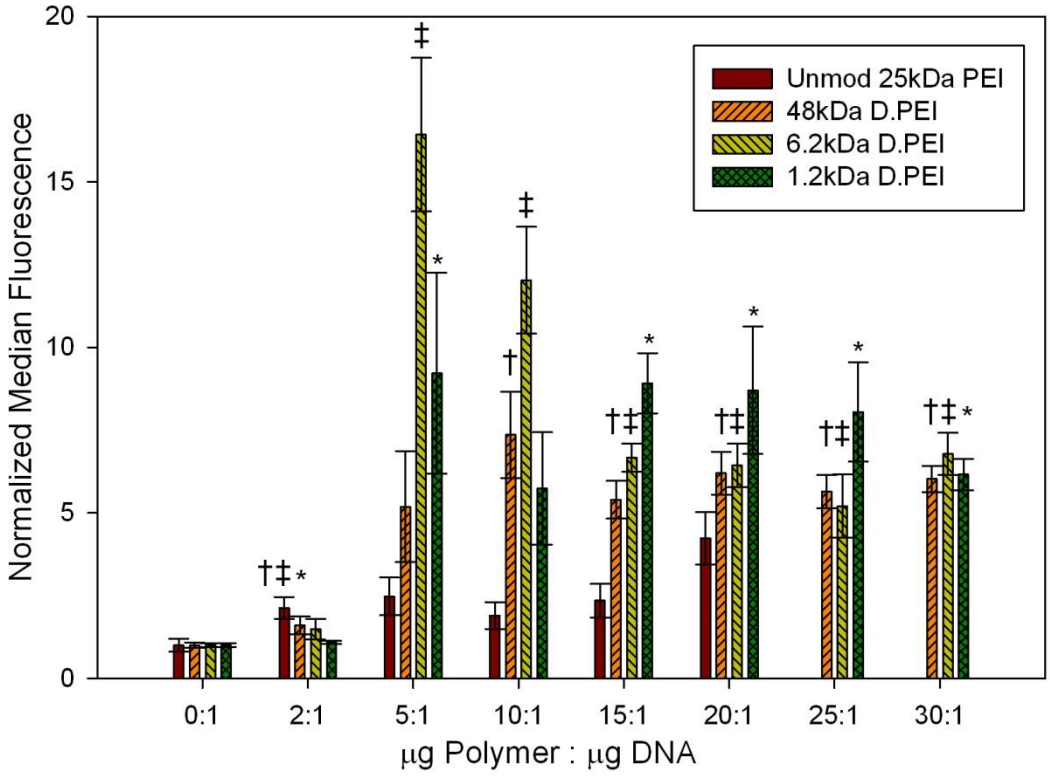

Figure 3.13: Cellular uptake of fluorescently labeled polyplexes with YOYO-1 intercalated pGL3 in MDA-MB-231 cells (t-test: †, $p<0.01$; ‡, $p<0.01$; *, $p<0.01$). Results are reported as median fluorescence normalized by control with no polymer present using FACS (N=3, error bars represent standard deviation).

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Chapter 4

Discussion, Conclusions, and Future Work

4.1 Discussion

Safety and efficacy are, quite simply, the most important factors in gene therapy. Compared to viruses, non-viral gene delivery vectors such as polymers and lipids are safer alternatives, but they display much lower efficiency and higher cytotoxicity. Much research has been done in optimizing polymers and lipids as potential delivery vehicles by chemically modifying their structures or by conjugating ligands onto their surfaces [1-3]. Specifically, the addition of degradable cross-linker in PEI has been extensively investigated in order to reduce the cytotoxicity and increase the transfection efficiency of PEI [4-5, 7]. Instead of synthesizing PEI derivatives with new cross-linkers and chemistry, we have further characterized and optimized the PEI derivatives first reported by Forrest et al. due to their relatively simple chemistry and efficient gene transfer [5].

Utilizing a heterogeneous PVP-supported Fe(III) catalyst, we synthesized a set of three D.PEIs with a wide range of molecular weights (1.2-48 kDa) through Michael addition between 800 Da PEI and 1,6-hexanediol diacrylate, resulting in D.PEIs with about a 15% cross-linking density (**Table 3.2**). NMR has confirmed the presence of ester linkages in each D.PEI and the degradation of the D.PEI when incubated at 37^{o}C (**Figure 3.2**, **3.3**, **and 3.4**). Both gel retardation and ethidium bromide exclusion studies indicated that all the D.PEIs are able to bind to DNA as tightly as unmodified 25 kDa PEI (**Figure 3.5** and **3.6**). At their optimal transfection ratios, the D.PEI polyplexes are ~1.4-fold larger than the unmodified 25 kDa PEI polyplexes before incubation, probably due to aggregation and the fact that the optimal D.PEI/DNA ratios for transfection are about 7-12-fold higher than that of 25 kDa PEI (**Table 3.3**). However, after 4

hours incubation, which simulates the 4 h exposure of polyplexes to DMEM during transfection, the polyplexes reached approximately the same size between D.PEIs and 25 kDa PEI, except for 1.2 kDa D.PEI polyplexes. This indicates that during transfection, the polyplexes swelled to similar size, making it unlikely that polyplex size plays a major factor in the improved transfection efficiency of the D.PEIs.

All three D.PEIs have demonstrated about 2-5-fold better transfection efficiency than unmodified 25 kDa PEI, in both HeLa and MDA-MB-231 cell lines, at their respective optimized transfection ratios (**Figure 3.8** and **3.9**). In particular, the higher molecular weight D.PEIs, 48 kDa and 6.2 kDa, consistently transfect better than 1.2 kDa D.PEI in both cell lines, which is in agreement with findings that suggest improved transfection efficiency with increasing PEI molecular weight [6]. However, the fact that 48 kDa D.PEI needs about 5-7-fold more polymer in order to transfect better than unmodified 25 kDa PEI indicates that in addition to molecular weight, there are other factors that contribute to the D.PEIs' improved transfection efficiency, likely involving cytotoxicity, cellular uptake, and DNA unpackaging.

Additionally, we have demonstrated the D.PEIs possess much lower cytotoxicity than unmodified 25 kDa PEI. D.PEIs show almost no toxic effect in MDA-MB-231 cells; 48 kDa and 6.2 kDa D.PEIs show about 50% viability, while 1.2 kDa D.PEI shows no toxic effect, in HeLa cells at 50 μg polymer/mL (**Figure 3.10** and **3.11**). The optimal transfection ratio for 25 kDa PEI is 2:1 μg polymer/μg DNA, which is approximately equal to a concentration of 2 μg polymer/mL. This concentration corresponds to about 60% and 80% viability for HeLa and MDA-MB-231 cell lines. Unlike unmodified PEI, our D.PEIs show above 80% viability in HeLa cells and almost no toxicity in MDA-MB-231 cells at their respective optimal transfection ratios. These results partially explain why, as we increased the amount of polymers used to complex with DNA, the transfection efficiency improved for D.PEI but regressed for unmodified PEI.

As discussed previously, cellular uptake and DNA unpackaging are two important aspects of gene delivery. Polyplexes must be able to enter the cell and release their DNA in order for the DNA to travel across the nuclear membrane or to be transcribed. HeLa cells show approximately the same cellular uptake for both unmodified PEI and D.PEIs, except for 48 kDa D.PEI, while MDA-MB-231 cells show 2-7-fold higher uptake of D.PEIs than unmodified PEI (**Figure 3.12** and **3.13**). The significant difference in cellular uptake between D.PEIs and unmodified PEI in MDA-MB-231 cells is in good agreement with the gene transfer trend we observed in the same cells. However, this is not the case for HeLa cells, where there is no discernible difference in uptake to correlate with the increase in transfection. To explain this discrepancy, we investigated the polymer's ability in unpackaging DNA through heparan sulfate competitive displacement assay. At the same polymer:DNA weight-to-weight ratio, D.PEIs show they more readily dissociate from DNA than unmodified 25 kDa PEI in the presence of heparan sulfate (**Figure 3.5**), which likely contributes to the increase in transfection efficiency for D.PEIs in both HeLa and MDA-MB-231 cells. These results show that gene delivery is a dynamic process where one must take various cellular barriers into account when designing potential non-viral gene delivery vectors.

Compared to the biodegradable PEI study first reported by Forrest et al. [5], this present work has further characterized the D.PEI and investigated the effects of molecular weight on D.PEI's gene delivery. Forrest et al. synthesized 14kDa and 30 kDa biodegradable PEIs by cross-linking 1,3-butanediol diacrylate and 1,6-hexanediol diacrylate, respectively, using 800 Da PEI without heterogenous catalyst and under different reaction conditions than that reported here. Forrest et al. showed that their D.PEIs were capable of condensing DNA, maintained reduced toxicity, and improved transfection efficiency in different cell lines but were unable to make any conclusive remarks on why their D.PEIs are more effective than the 25 kDa control or the effect of D.PEI's molecular weight on gene transfer abilities. However, in this current report, we synthesized

D.PEIs with a wide range of molecular weights, which has allowed us to correlate D.PEI's molecular weight with various gene delivery properties of cross-linked PEI. Specifically, we are able to explain the improved transfection efficiency through the differences between D.PEIs and unmodified PEI in terms of molecular weight, cytotoxicity, polyplex internalization, and DNA unpackaging ability.

As mentioned in the **Introduction**, several groups have reported various diacrylate cross-linked PEIs and show at least 50-fold improvement in transfection efficiency and reduced cytotoxicity compared to the controls [8-11]. However, it is difficult to draw a direct comparison between those polymers and the polymers reported here, mainly due to the use of different cells and controls. In addition, many of those reports only studied how the size, degradability, and cytotoxicity of the cross-linked PEI polyplexes affect transfection but neglect the effects of intracellular barriers on gene delivery, namely cellular uptake and DNA unpackaging of the polyplexes. Without this characterization information, it is impossible to fully understand or compare any cross-linked PEIs as polymeric gene delivery vectors and to explain relative enhancement in gene transfer ability.

4.2 Conclusions and Future Work

We reported here a controllable synthesis of biodegradable PEI by cross-linking 800 Da PEI and 1,6-hexanediol diacrylate through the use of a polymer-supported heterogeneous catalyst. The resulting polymers have a wider range of molecular weights and have demonstrated several fold more efficient gene transfer compared to commercially available, unmodified, 25 kDa PEI. The improvement in gene delivery of these D.PEIs is likely due to some combination of their low cytotoxicity, easy DNA unpackaging, and high cellular uptake.

Future studies should examine the intracellular trafficking mechanisms of D.PEIs in both of the cell types utilized in the present study. This will enable us to understand the fundamental cellular behaviors driving the improved performance of our D.PEIs in order to optimize the design of future PEI-based materials. Specifically, we will investigate the D.PEIs' cellular uptake, such as clathrin- or caveolin-mediated pathways, by utilizing appropriate targeting ligands and drugs. Additionally, we will study the effect of endosomal escape by disrupting the endosome and observe any effects on the D.PEIs' gene transfer abilities. Pending the success of this additional in vitro characterization, we may then proceed to in vivo testing due to the enormous therapeutic promise of our described D.PEIs

4.3 References

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Author's Biography

Wing Tat Victor Shum was born on October 8th, 1983, in Hong Kong. He and his mother immigrated to the U.S. in 1998 and have lived in Oakland/San Leandro, CA ever since. In 2002, Victor completed his high school education at Chinese Christian School in San Leandro, CA. Upon receiving his B.S. in Chemical Engineering at the University of California, Berkeley, CA, in December 2006, Victor began his graduate study at the University of Illinois at Urbana-Champaign, IL, in August 2007.